VACCINIA VIRUS TRANSCRIPT RELEASE REQUIRES THE VACCINIA VIRUS PROTEIN A18 AND A HOST CELL FACTOR

By

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2000

This work is dedicated to the memory of my grandfather, Joseph Vavrik.

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Abstract of Dissertation Presented to the Graduate School
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VACCINIA VIRUS TRANSCRIPT RELEASE REQUIRES THE VACCINIA VIRUS PROTEIN A18 AND A HOST CELL FACTOR

By

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Major Department: Molecular Genetics and Microbiology

Elongation and termination have proven to be important stages in the regulation of both prokaryotic and eukaryotic transcription. Vaccinia virus is an extremely useful model for the study of gene expression because the virus encodes much of its own enzymatic transcription machinery. Genetic studies have identified several viral proteins that are believed to be involved in vaccinia virus transcription elongation and termination. The *in vivo* analysis of viruses containing mutations in the genes *G2R* and *J3R* indicate that the transcripts synthesized from intermediate and late genes are 3' truncated as compared to a wild type (Wt) infection. We hypothesize that G2 and J3 function as positive transcription elongation factors. Prior phenotypic analysis of a vaccinia virus gene *A18R* mutant, Cts23, showed the synthesis of longer than Wt length viral transcripts during the intermediate stage of infection, indicating that the A18 protein may act as a negative transcription elongation factor. The overall goal of the research described here is to provide a biochemical characterization of the regulation of vaccinia

virus transcription elongation and/or termination. Pulse-labeled transcription complexes established from intermediate viral promoters on bead-bound DNA templates were assayed for elongation and transcript release during an elongation step that contained nucleotides and various proteins. The addition of Wt extract during the elongation phase resulted in release of the nascent transcript as compared to extract from Cts23- or mock-infected cells that were unable to induce release. The lack of release following addition of Cts23 extract suggests that A18 is involved in the release of nascent RNA. By itself, purified polyhistidine-tagged A18 protein (His-A18) was unable to induce release; however, release did occur in the presence of purified His-A18 protein plus extract from Cts23- or mock-infected cells, suggesting that an additional factor(s) is present in uninfected cells. These data taken together indicate that A18 is necessary but not sufficient for release of nascent transcripts. To identify the cellular factor(s), purification using conventional chromatography was initiated. We conclude that A18 and an as yet unidentified cellular factor(s) are required for the *in vitro* release of nascent RNA from a vaccinia virus transcription elongation complex.

CHAPTER 1
INTRODUCTION

Overview of Eukaryotic and Prokaryotic Gene Expression

The synthesis of messenger RNA is regulated at all stages from pre-initiation to termination and 3'-end processing. This control ensures the appropriate expression of the multitude of genes necessary during the life cycle of any organism. Prokaryotic gene expression is regulated at the level of initiation by activators and repressors, such as in the lac operon, and during promoter clearance, elongation, and termination. Eukaryotic gene expression also is regulated at many levels including chromatin remodeling, promoter activation, transcription complex assembly, promoter clearance, elongation, termination, and mRNA processing. Recent work in the field of transcription identified many higher order complexes that indicate an interaction among all of the factors regulating transcription.

This introduction includes descriptions of the stages and factors involved in gene expression of prokaryotes, yeast, and metazoans. The bacterial polymerase and its associated factors are highly amenable to both *in vitro* biochemistry using purified components and genetic analysis. The prokaryotic system is much simpler than its eukaryotic counterparts and for this reason is subject to a high degree of study. Yeast, the model eukaryote, is still a relatively simple system subject to genetic analysis but with a more complex regulation than prokaryotes. The detailed knowledge ascertained from studies with prokaryotes and yeast can be applied to metazoans that have a much more

complex biochemistry and limited genetics. Vaccinia virus synthesizes eukaryotic-like mRNA but due to its cytoplasmic life cycle has evolved less complicated transcription machinery. This affords us an ideal system for the study of mechanisms involved in the regulation of transcription elongation and termination. The state of the art in transcription is summarized here with a specific focus on the mechanisms and factors involved.

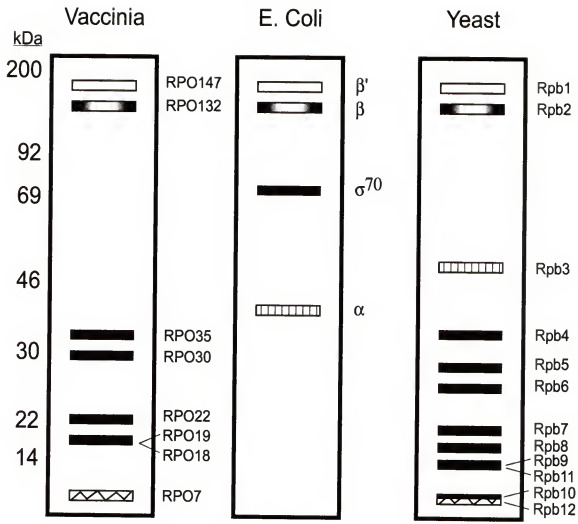
RNA Polymerase

The RNA polymerase is the primary component of all transcription complexes on which the other factors build. The core RNA polymerase is defined as the minimal set of elements required for promoter-independent transcription on a DNA template *in vitro*. The bacterial core enzyme is composed of two α subunits, and a single β and β' subunit ($\alpha_2\beta\beta'$). Using an assay for promoter-specific transcription, the σ factor was discovered. The σ factor associates with the core RNA polymerase in the absence of promoter DNA to form a holoenzyme complex ($\alpha_2\beta\beta'\sigma$) that is now capable of promoter recognition and transcription initiation (19,72). Several forms of σ factor have been identified, although σ^{70} is the principal factor used by most promoters. The alternative σ factors direct the RNA polymerase to structurally distinct promoters and control genes for specialized functions such as the heat shock response, expression of flagellar and chemotaxis genes, and control of nitrogen metabolism (56-58,75). A high-resolution crystal structure of the *Thermus aquaticus* (Taq) RNA polymerase will be discussed later in this introduction.

In eukaryotes, three DNA-dependent RNA polymerases (designated I, II, and III) transcribe ribosomal genes (rRNA), protein-coding genes (mRNA), and genes coding for tRNA and other small RNAs, respectively. This description concentrates on RNA

polymerase II (RNAPII), where most of the work on transcription has focused, although important insights are also derived from work on RNA polymerase I (RNAPI) and RNA polymerase III (RNAPIII) and are discussed briefly. Similar to prokaryotes, RNAPII was first purified using promoter-less template transcription assays (136). Both yeast and human RNAPII are composed of 12 similar subunits, among which there is extensive structural conservation. These 12 subunits comprise the equivalent of the prokaryotic core enzyme. The two largest subunits, Rpb1 and Rpb2, are the most highly conserved and are homologous to the β' and β subunits, respectively, of bacterial RNA polymerase (Fig. 1). The Rpb3 subunit is related to the α subunit of bacterial RNA polymerase. Although none of the RNAPII subunits are closely related to the σ subunit, the general transcription factors (GTFs) of RNAPII are the functional counterparts (111). The GTFs are discussed in more detail below. A unique feature of the largest RNAPII subunit, Rpb1, is a highly conserved domain consisting of 26 to 52 repeats (depending on the species) of the consensus sequence YSPTSPS at the carboxy-terminus (CTD). The CTD is not present in the prokaryotic β' subunit, the related subunit of RNAPI or RNAPIII, or the RPO147 subunit of vaccinia virus RNA polymerase. The deletion of most or all of the CTD in yeast is lethal, demonstrating that the domain is essential *in vivo*. An RNAP containing a hypophosphorylated CTD is recruited to the pre-initiation complex and at some point during the transition from initiation to elongation the CTD becomes highly phosphorylated. Several cellular kinases are implicated in this event including the initiation factor TFIIH and the positive elongation factor P-TEFb. The role of these factors will be described in more detail below.

Fig. 1. **RNAP subunit composition from vaccinia virus, *E. coli*, and *S. cerevisiae.*** The cartoon represents the separation of RNAP subunits after SDS polyacrylamide gel electrophoresis indicating the apparent molecular size of each subunit. The size in kDa is indicated at the *left*. Sequence, amino acid, and/or functional homologies between subunits of different species are indicated by similar fill patterns. The subunits shown in black do not have significant homology. This cartoon was adapted from Woychik and Young (158).


Vaccinia
E. Coli
Yeast
kDa
200
92
69
46
30
22
14
RPO147
RPO132
RPO35
RPO30
RPO22
RPO19
RPO18
RPO7
β'
β
σ70
α
Rpb1
Rpb2
Rpb3
Rpb4
Rpb5
Rpb6
Rpb7
Rpb8
Rpb9
Rpb11
Rpb10
Rpb12

There is increasing evidence for an RNAPII "holoenzyme" as indicated by the isolation of various multiprotein complexes interacting with core RNAPII. For recognition and promoter-specific initiation, core RNAPII requires a set of additional proteins known as the general transcription factors (GTFs) similar to the requirement for σ factor in prokaryotes. The GTFs include TFIID, TFIIB, TFIIE, TFIIF, and TFIIH. In yeast and mammals, the five GTFs together comprise a total of 23 polypeptides. Although purified RNAPII and the GTFs are sufficient for promoter-specific initiation, they fail to respond to activators *in vitro*, implying the necessity of another factor. First, several proteins designated as Srb proteins were identified during a selection for second-site suppressors of a partial truncation of the CTD in yeast Rpb1. Second, a multi-protein complex from yeast termed "mediator" was identified based on its ability to mediate activation (45). A less complex "core" Srb-mediator complex recently was purified (105) and includes a subset of Srb proteins, a subset of mediator proteins denoted MEDs, and several polypeptides previously identified as positive and negative effectors of transcription (102). The exact number and composition of the polypeptides of the Srb-mediator complex differs based on the method of purification and functional requirements imposed. For example, in addition to the aforementioned polypeptides, a subcomplex consisting of four Srb proteins is required for repression at some repressors (101). Regardless of the precise composition, the function of the Srb-mediator complex is mediated through interactions with the RNAPII CTD (102). An additional complex, isolated using an antibody to the CTD of RNAPII, lacks Srb and mediator subunits but includes a subset of the GTFs. It is possible that the Srb-mediator interaction with the CTD was disrupted by the CTD antibody (141). Two attempts at purification of human

RNAPII complexes using conventional chromatography have resulted in different complex composition as well. Analysis of polypeptides that co-elute with RNAPII identified a complex containing chromatin-remodeling activities, including the SWI/SNF and histone acetyltransferases CBP and PCAF, but was lacking the GTFs. This complex was not assayed for transcription activity (25). The second purification involved chromatography fractions that were assayed on naked DNA templates. This resulted in the isolation of a complex lacking the chromatin-remodeling factors but including a subset of Srb-mediator proteins and GTFs (86). These data support a model of RNA holoenzyme that contains many subcomplexes important for mediating a number of regulatory events. A model for transcription complex formation based on these data is discussed below.

Transcription Initiation

For the purpose of this dissertation, descriptions of several pre-initiation events are grouped with those involved in initiation. The mechanism of prokaryotic transcription initiation is less complex than that of eukaryotes due to the fact that the prokaryotic template is not packaged in nucleosomes and there are fewer protein factors involved. Specific and accurate eukaryotic transcription initiation is more complex and requires chromatin remodeling, promoter activation, pre-initiation complex (PIC) assembly, and initiation.

Prokaryotic transcription initiation

Prokaryotic transcription initiation is accomplished in several steps. The core RNAP ($\alpha_2\beta\beta'$) first associates with the σ factor in the absence of DNA to form the RNAP holoenzyme. The holoenzyme binds to the promoter DNA to form an RNAP-promoter

closed complex. The σ^{70} factor binds to each of the core RNA polymerase subunits and can only bind DNA when complexed with the core enzyme. The structure of the polymerase suggests that the "jaw-like" clamp of the RNAP holoenzyme is capable of clamping down on the promoter DNA to yield an RNAP-promoter intermediate complex. The double-stranded DNA strands are melted to reveal 12 to 14 nt of open DNA surrounding the transcription start site. The formation of this RNAP-promoter open complex allows access to the genetic information in the template strand of the DNA (40,95). Both repressors and activators control the regulation of transcription initiation. Repressors function either by physically blocking the RNA polymerase or by forming a repressosome structure. Activators stimulate transcription by direct interaction with at least three of the subunits of the RNA polymerase (α, σ, β'). Activators can either increase the association of the polymerase with the promoter or stimulate the RNA polymerase activity (148).

Eukaryotic chromatin remodeling

The coiling of DNA around a histone octamer to form a nucleosome provides a major stage of transcriptional regulation in eukaryotes. A nucleosome consists of two copies each of four histone proteins, H2A, H2B, H3, and H4, which interact with the DNA to form a core particle. Each histone also possesses an amino-terminal tail that extends outside of the core particle, a prime target of interaction for higher-ordered coiling and gene activation. The result is a highly ordered structure that is capable of repressing transcription. This was demonstrated by depletion of histone H4 in yeast, which prevented the formation of intact nucleosomes and led to activation of several promoters including *PHO5* (53).

How does the transcription machinery deal with the chromatin template? Nucleosomes prevent binding of TBP, a subunit of TFIID, to the TATA promoter element *in vitro* (74), and yet have only a slight inhibitory effect on the ability of a variety of activator proteins to bind their target sites. Eukaryotic activators could enhance the recruitment of RNAPII to its promoter by either a direct interaction of activators with components of the polymerase or an indirect recruitment of RNAPII by alteration of the chromatin structure. Recent work delineating the RNAP holoenzyme revealed the presence of activator proteins that interact with proteins possessing catalytic activities directed at the histones. As previously described, one purified human holoenzyme complex contained SWI/SNF, a chromatin remodeling complex, and CBP, a histone acetylase. Histone acetylation is a characteristic of transcribed chromatin (25). Several chromatin-remodeling complexes were identified from different organisms and include SWI/SNF from *Drosophila,* yeast, and humans; RSC (remodels the structure of chromatin) from yeast; and CHRAC (chromatin accessibility complex) from *Drosophila* (20). Recently the use of an *in vitro* abortive initiation assay on a chromatin template revealed two additional human chromatin-remodeling complexes; RSF (remodeling and spacing factor) and ACF (ATP-utilizing chromatin assembly and remodeling factor) that promote initiation only in the presence of an activator (79,80). These two complexes are independent yet both contain the hSNF2h protein. The larger subunits are unique to each complex and must be responsible for specificity. The combination of activators and a chromatin-remodeling complex may open the chromatin template near the promoter to achieve transcription initiation. However, this does not necessitate the removal of all the nucleosomes for a given transcribed gene. The polymerase complex must interact further

with the chromatin template as elongation continues and this is addressed in a subsequent section.

Eukaryotic pre-initiation complex assembly

The necessary components of the promoter region were defined by mutational analysis. The structure of eukaryotic promoters is divided into two portions: the core promoter of approximately 50-bp adjacent to the transcription start site and a more distant enhancer region (129,154). The core promoter elements are defined as "the minimal DNA elements that are necessary and sufficient for accurate transcription initiation by RNAPII in reconstituted cell-free systems" (106). The core promoter consists of a TATA box located near position -30 to -25 and a pyrimidine-rich initiator (Inr) region located near the transcription start site (position +1) (106). The enhancer is important for interaction with activator proteins and can be located either upstream or downstream of the core promoter (148).

Order of addition experiments demonstrated that purified general transcription factors (GTFs) assemble at the promoter in a stepwise manner *in vitro*. Transcription complex assembly at the promoter is initiated by TFIID via the TBP subunit binding to the TATA element of the promoter, followed by binding of TFIIB that in turn recruits RNAPII-TFIIF, TFIIE and TFIIH (111). This work was essential for establishing a basic understanding of the interactions between the GTFs. However, this assay does not necessarily mimic the *in vivo* situation in terms of factor ratios and preassembled complexes.

Using an immobilized template assay and nuclear extract, which more closely resembles the *in vivo* situation, two pre-initiation complex (PIC) intermediates were isolated (122). A new model of PIC assembly is based on these data. The first step in

assembly is TBP binding to the TATA box along with TFIIA. The transcription factor TFIIA was shown *in vivo* and *in vitro* to "encourage" the interaction between TFIID and the promoter (30). The second intermediate is composed of RNAPII, TFIIB, the Srb4 protein (a component of the Srb-mediator complex), TFIIE, and TFIIH (122). The identification of this intermediate suggests that the polymerase enters the PIC bound to the GTFs (except TFIID) and to the Srb-mediator complex.

Eukaryotic initiation

The initiation of transcription is signified by formation of the first phosphodiester bond. The functions of the yeast GTFs in initiation were studied by analysis of mutant subunits *in vitro* and *in vivo*. The GTF TFIIB directly interacts with TBP and the DNA sequence surrounding the TATA element to recruit the RNAPII holoenzyme complex to the promoter (104) and also affects transcription start site selection (81). Genetic and biochemical studies indicate that TFIIF interacts directly with TFIIB and helps stabilize RNAPII at the promoter. The TFIIH factor has an ATP-dependent DNA helicase activity that is required for promoter melting, and a kinase activity that was shown to phosphorylate a number of targets including the CTD of RNAPII (151). The phosphorylation state of the CTD is linked to the transition from initiation to elongation. The transition from a closed to an open complex is ATP-dependent and thought to be preceded by phosphorylation of various sites in the initiation complex including the CTD. After promoter clearance, TBP remains at the promoter, TFIIF remains bound to the polymerase, and the other GTFs are released. There is also an indication of an exchange of the Srb-mediator complex for another multisubunit complex called Elongator, a step that may be linked to the phosphorylation state of the RNAPII CTD (114).

Transcription Elongation

Elongation is the process by which RNAP catalyzes the successive polymerization of nucleotide monophosphates into an RNA transcript based on their complementarity to bases in a DNA template. The central component of elongation is the ternary complex composed of the RNAP, the template DNA, and the nascent transcript. Most of what is known about elongation derives from studies of bacterial RNAP. The known biochemical data derived from *E. coli* are supported by the recent elucidation of a high-resolution crystal structure for Taq RNAP. This is an exciting time for the elongation field, as many hypotheses regarding conformational changes that the polymerase undergoes to achieve elongation can begin to be supported by structural data. This dissertation provides a summary of these data and a discussion of the stages of transcription elongation. These stages include promoter clearance, the structure of the RNAP ternary complex, a description of backtracking, and a summary of some of the known elongation factors.

Promoter clearance

Entry into the stage of transcription defined as elongation requires that the transcription complex clear the promoter. In prokaryotes, the transition from initiation to elongation is characterized by three biochemical changes in the RNAP complex: the RNAP undergoes the first translocation that displaces the polymerase relative to the promoter, the σ factor is released, and the ternary complex becomes tightly associated with both the DNA template and the nascent RNA resulting in a very stable complex (155). The polymerase also undergoes a process defined as abortive initiation that generates a set of nested RNA transcripts that are less than 12-nt in length. This process may reflect multiple attempts of the RNAP to direct the 5'-end of the growing RNA chain

to the RNA binding site of the polymerase. The placement of the RNA 5'-end is not understood but it is recognized as key to rendering the complex fully stable. In *E. coli* this process coincides with release of the σ subunit after the synthesis of between 4 and 10-nt and may reflect a conformational change of the RNAP to an elongation competent form. The conformational change on transition from initiation to elongation is supported by a nearly two fold decrease in the footprint size of the polymerase (107).

Eukaryotes have analogous reactions for promoter clearance. The transition from initiation to elongation requires breaking the initial ties with the promoter, the GTFs, and the accessory factors, as well as conversion of RNAP to an elongation competent form. Promoter clearance is also plagued by abortive initiation and arrest. The RNAPII complexes containing transcripts less than 9-nt in length are unstable and likely to abort (59,66). Dissociation of the GTFs occurs after the synthesis of 10- to 15-nt of RNA. The transition from initiation to elongation is accompanied by an increase in the stability of the ternary complex. This stability is necessary for the processivity of the RNAP based on the fact that if the polymerase releases the nascent RNA prior to complete synthesis it is unable to rebind and continue transcription. The synthesis of new RNA must begin with reinitiation at the promoter. Most elongation complexes are stable in high concentrations of salt, survive purification by gel filtration or precipitation with an antibody, and can be stored in the absence of nucleotides at 4°C for days without significant loss of activity (155).

Many proteins are implicated in the regulation of eukaryotic early elongation. The general transcription factor TFIIF is required for initiation and stimulation of elongation and may act to decrease abortive initiation by increasing the rate of nucleotide

addition (167). As previously described, the transition from initiation to elongation is accompanied by opening of the DNA template and phosphorylation of the CTD of RNAPII, events that could be accomplished by the TFIIH ATPase, helicase, and kinase activities. Additional positive and negative transcription elongation factors (P-TEFs and N-TEFs) are postulated to regulate promoter clearance in a DRB-sensitive manner (26,89). Several of these factors were identified including P-TEFb, NELF, DSIF, and Factor 2. P-TEFb is the positively acting factor that is composed of Cdk9 and cyclin T1. Although the kinase activity of P-TEFb could have more than one target for phosphorylation, evidence suggests that the CTD of RNAPII is a physiologically important target (88). The phosphorylation of the CTD by P-TEFb is required to prevent arrest by the elongating RNAPII. The NELF and DSIF factors interact with an RNAPII containing a hypophosphorylated CTD to negatively regulate elongation (166). Factor 2 is responsible for the release of short transcripts from early elongation complexes (165). These negative activities may be overcome by the positive action of P-TEFb (156,166).

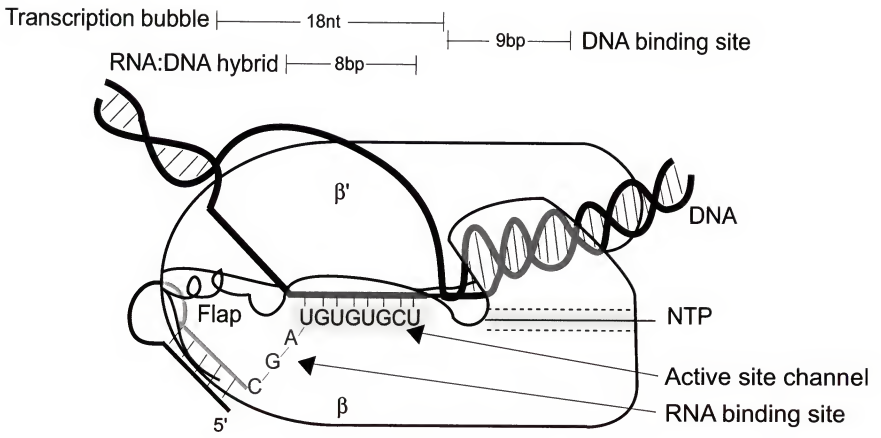
Current model of the structure of the RNAP ternary complex

Analysis using X-ray crystallography has revealed the structure of *Thermus aquaticus* (Taq) RNA polymerase (RNAP) at 3.3 A resolution (169). The Taq RNAP is similar in size and shape and has a high degree of sequence similarity to the *E. coli* RNAP defined by low-resolution electron crystallography (36,37). This allows for the comparison of the biochemical data elucidated from work with *E. coli* and the structural data from Taq to construct a structure-function model of the transcription complex. The RNAP has a "crab-claw-like" shape where the "jaws" are separated by several channels leading to the Mg^{+2} active site (Fig. 2). It is suggested that the "jaws" close around the downstream duplex DNA in the transition from initiation to elongation. One channel

encloses the double-stranded DNA downstream of the transcription bubble, while a second channel accommodates the upstream DNA resulting in a 90° bend of the DNA. An upstream element called the "rudder" protrudes from the floor of the active site and is positioned such that it could separate the DNA template strand and the RNA transcript thus allowing the two strands of DNA to reanneal. Crosslinking studies predict another channel for the passage of the RNA transcript opposite the DNA. An additional channel called the secondary channel is postulated to recruit nucleotides and may be blocked by the RNA 3'-end when in the backtracked conformation (169).

Three sites in the elongation complex were characterized functionally and structurally: the double-stranded DNA binding site, the RNA-DNA heteroduplex binding site, and the single-stranded RNA binding site (Fig. 2) (107). The DNA binding site was mapped to 9-bp of double-stranded DNA just downstream of the 18-nt transcription bubble (108). The RNA-DNA hybrid is composed of 8-bp as determined by chemical footprinting and RNA-DNA chemical cross-linking (109). The heteroduplex-binding site is a region of weak ionic interactions between the protein and the first six basepairs of the RNA-DNA hybrid. The RNA binding site was defined using photoreactivated RNA probes that showed tight RNA contacts with the RNAP and nine nucleotides of RNA spanning from -8 to -17 next to the hybrid-binding site. Footprint analysis shows protection of 40- to 50-bp of DNA (124) and 18-nt of RNA (49) in the transcription elongation complex.

Fig. 2. **Model of the paused transcription elongation complex.** The cartoon represents the structure-function model of the prokaryotic polymerase based on structural data from Taq and biochemical data from *E. coli*. The cartoon is adapted from the meeting notes from 'Post-initiation Activities of RNA Polymerase', Fall 2000 meeting, Mountain Lake, Virginia and Mooney, Artsimovitch, and Landick (95).


Transcription bubble
18nt
9bp
DNA binding site
RNA:DNA hybrid
8bp
β'
DNA
Flap
UGUGUGCU
NTP
A
G
C
Active site channel
RNA binding site
β
5'

Backtracking of the ternary complex

The study of the mechanistic aspects of transcription elongation is confined to work in prokaryotes due to the simplicity of the RNAP enzyme and the difficulty of the elongation assays. There are three blocks to transcription elongation at which factors theoretically can act to effect elongation: pause, arrest, and termination. Transcription pause and arrest can be a result of intrinsic signals (interactions between the RNAP and sequence in the RNA and DNA), a response to DNA binding proteins that physically block progression of the RNAP, or a response to artificial conditions such as the absence of one of the four nucleotides. Pausing is a temporary delay in RNA chain elongation and is a precursor to arrest (complete halting without dissociation) and dissociation of the ternary complex at ρ-independent and ρ-dependent terminators (126). However, not all pauses are termination precursors (155).

The relationship between translocation of the RNAP and synthesis of each phosphodiester bond of the RNA transcript is a subject of great debate. Several models were proposed over the past seven years including the classical and revisionist models. In the classical model, the RNAP moves along the template monotonically, i.e., the RNAP moves synchronously with the addition of each nucleotide (46). In the revisionist model, or the inchworm model, the RNAP is proposed to move in a two-step cycle. The RNA is synthesized while the RNAP is in a static position, and movement of the RNAP occurs in short bursts, or jumps, which allows the DNA and RNA to be threaded through the enzyme (21). The inchworm model was based on three lines of experimental evidence: irregular DNA footprints of elongation complexes halted at successive sites

(73), formation of arrested transcription complexes (3), and cleavage of internal RNA from defined complexes resulting in the loss of 3' RNA fragments (150).

Recent data indicate that the inchworm model of contraction and expansion of the RNAP was a misinterpretation. Footprinting experiments have now demonstrated that a stalled *E. coli* RNAP translocates backwards relative to the catalytic site and that the translocation can be suppressed by hybridization of oligonucleotides upstream of the RNAP (69). This activity is described as backtracking, or the lateral oscillation of the RNAP ternary complex. Backtracking also was demonstrated using nucleotide analogs that either strengthen or weaken the RNA-DNA hybrid (109). Pause, arrest, and termination signals all appear to slow RNAP due to unstable basepairing in the hybrid that displaces the RNA 3'-end from the active site of the RNAP. This instability may allow the backward sliding (backtracking) of RNAP into a more stable complex. The backtracking model may provide an explanation for transcriptional fidelity and control of the rate of transcription elongation.

Recent work from Landick and co-workers has demonstrated that the elongating RNAP can adopt open and closed conformations that dictate slow and fast elongation by the RNAP (39). These conformational changes are suggested by the structure of the RNAP, kinetic studies of RNAP, and formation of a pause RNA hairpin in prokaryotes. The flexibility and structure of the RNAP suggests that the jaws of the RNAP may close around the DNA, locking the downstream double-stranded DNA within the DNA binding site. In addition, a flap of the RNAP is predicted to close over the exiting transcript thus creating the RNA binding site (169). These two modifications of the polymerase create a closed conformation that is capable of rapid elongation. This idea is supported by the

kinetics of elongation observed on single RNAP molecules that reveal dynamics that are averaged out in bulk RNAP experiments. The kinetics suggest both a fast and slow state of incorporation of nucleotides (39).

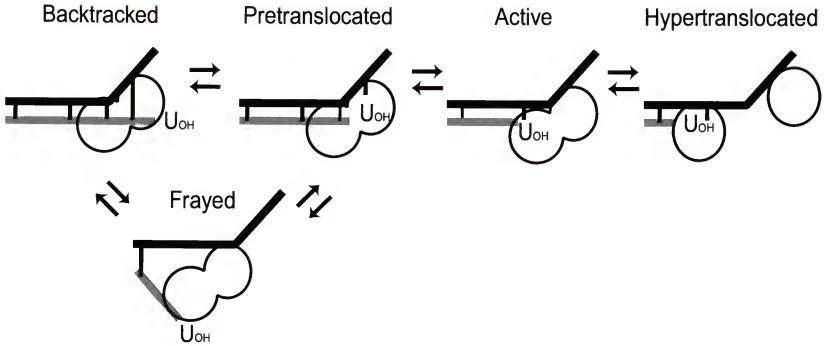
RNA hairpins that form as the transcript emerges from the ternary complex are integral parts of some pause signals and are required at ρ-independent terminators where they induce dissociation of the ternary complex. RNA hairpins can also function as antiterminators which alter the ternary complex to block recognition of both pause and termination sites. The role of hairpin formation in termination and antitermination is discussed in more detail below. Mutational analysis of the pause hairpin indicates that the structure and not the specific sequence within the hairpin are important for pausing (23). It should be noted that hairpin formation is not sufficient to signal a pause, and not all pauses contain stable RNA hairpins (82). DNA and RNA sequences between the base of the hairpin and the RNAP active site also affect pausing and this distance may, in part, distinguish ρ-independent termination from transcription pause sites (23). The use of crosslinking agents demonstrated that the loop region of an RNA hairpin makes contacts with the RNAP flap, the same structure that is predicted to close over the exiting RNA (Fig. 2). The interaction with the RNA hairpin may open the grasp of the RNAP flap on the exiting RNA. Based on the structure of the RNAP, the flap is linked to the β' subunit which forms the base of the channel contacting the RNA-DNA hybrid. This link may explain how formation of an RNA hairpin can lead to disruption of the catalytic activity of the RNAP. These data together support the model of fast and slow elongation characterized by closed and open conformations, respectively, of the RNAP (5).

The Landick model also suggests that at every template position the RNAP can fluctuate between normal elongation and a state susceptible to pausing, arrest, or termination. The position of the RNA 3'-end may vary between several different positions including backtracked (RNA extending downstream of the active site), frayed (RNA 3'-end is separated from the template DNA strand), pretranslocated (RNA blocking the nucleotide binding site), active (RNA primed for nucleotide addition), and hypertranslocated (RNA 3'-end pulled out of the active site) (Fig. 3) (5). The RNAP switches between the active and pretranslocated conformations during rapid elongation and may engage in the other conformations at pause and arrest sites (5). Rescue from these conformations may be spontaneous or the result of regulation by specialized proteins. There are two highly studied types of pause signals that are characterized by the structure of the paused complex. These signals are designated as class I and class II pauses (5). A class I pause site is characterized by the interaction between an RNA hairpin and the RNAP but is also dependent on the 11-nt distance between the base of the hairpin and the 3'-end. These pauses are found in the leader regions of several bacterial amino acid biosynthetic operons. The interaction between the RNA hairpin and the RNAP induces the RNA 3'-end to adopt the frayed or hypertranslocated position (Fig. 3) (4,24). A class II pause is characterized by a weak RNA-DNA hybrid that induces backtracking of the RNAP (Fig. 3). These pause sites have been characterized *in vitro* at arrest or termination sites and in the early transcribed region of *E. coli* to recruit the antitermination factor RfaH.

Elongation factors

The factors that regulate transcription elongation can be divided into at least three functional classes based on their ability to prevent arrest of RNAP, to regulate the rate of

Fig. 3. **Position of the RNA 3' end at various positions.** The cartoon represents the possible positions of the RNA 3' end (U_{OH}) during active elongation and pausing. The active site of the RNAP is represented by the circles. The template DNA is indicated as a black line and the RNA as a gray line. This cartoon was adapted from Artsimovitch and Landick (5).


Backtracked
Pretranslocated
Active
Hypertranslocated
Frayed
UOH
UOH
UOH
UOH
UOH

RNAP through chromosomal templates, and to increase the catalytic rate through suppression of pausing. Factors that prevent arrest of the RNAP include the prokaryotic factors GreA and GreB, and the eukaryotic factors P-TEFb and SII (Table 1). The prokaryotic Gre factors and eukaryotic factor SII share functional but not sequence or structural similarities. These factors interact with their respective RNAPs to activate the endoribonucleolytic cleavage activity intrinsic to the polymerase at sites of DNA-specific arrest. Induction of the cleavage activity in a backtracked ternary complex (Fig. 3) removes the unpaired 3'-end of the nascent transcript and repositions the RNA in the polymerase active site for continued elongation (13,90,110). Unlike GreA, GreB and SII can act on a ternary complex that has arrested in the absence of the factor. GreA, on the other hand, must be associated with the ternary complex prior to arrest in order to activate the cleavage activity.

In eukaryotes, the production of full-length runoff transcripts *in vitro* and functional mRNA *in vivo* is sensitive to the drug 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). The *Drosophila* and human factor P-TEFb rescues DRB-sensitive arrest in early eukaryotic elongation complexes. It is composed of two subunits, a cyclin (T1, T2a, or T2b) and a cyclin-dependent kinase (Cdk9) (118,119). The P-TEFb factor can phosphorylate the CTD of RNAPII, an action that is thought to regulate the transition from abortive initiation to elongation. The action of P-TEFb also is postulated to counteract the actions of N-TEFs, negative transcription elongation factors, including DSIF and NELF (156,166).

Several eukaryotic factors were identified based on their ability to promote transcription through chromatin templates. An ATP-dependent activity found in fractions

Table 1: Select Prokaryotic and Eukaryotic Transcription Elongation and Termination Factors

Name	System	Function	Properties
DSIF	Eukaryotes, yeast	Induce arrest	Binds RNAPII *in vitro*, works with NELF to arrest RNAPII, activity is counteracted by P-TEFb
ELL	Eukaryotes	Stimulate elongation	Inhibits transcription initiation by competing for RNAPII, suppress transient pausing, suppress backtracking?, implicated in oncogenesis
Elongin	Eukaryotes	Stimulate elongation	Implicated in oncogenesis, suppress backtracking?
FACT	Eukaryotes, yeast	Histone chaperone	Stimulates transcription through chromatin
Factor2	Eukaryotes	Transcript release factor	DNA-dependent ATPase
GreA	Prokaryotes	Prevent arrest	Cleavage stimulatory factor, interacts with RNAP β subunit, must be associated with complex prior to arrest
GreB	Prokaryotes	Prevent arrest	Cleavage stimulatory factor, 35% aa identity with GreA, acts on arrested complex
N	Prokaryotes	Anti-termination	RNA binding protein, binds RNAP, induces read through of termination factor dependent and independent sites on λ genome
NELF	Eukaryotes	Induce arrest	Works with DSIF to arrest RNAPII, activity is counteracted by P-TEFb
NusG	Prokaryotes	Accelerates elongation	Interacts with core RNAP, stimulates escape from class II pause sites, inhibits backtracking?
P-TEFb	Eukaryotes	Prevent arrest	Stimulates the production of long transcripts, ATP-dependent, phosphorylates RNAPII CTD, counteracts N-TEFs
PTRF	Eukaryotes	RNAPI termination	Induces blocked murine RNAPI to terminate
Q	Prokaryotes	Anti-termination	DNA binding protein, promotes read through of termination signals
Reb1p	Yeast	RNAPI termination	DNA binding protein, blocks elongating yeast RNAPI
Rho	Prokaryotes	Termination	RNA binding protein, ATPase, RNA-DNA helicase
SII (TFIIS)	Eukaryotes, yeast	Prevent arrest	Stimulates NTP incorporation, binds RNAPII *in vitro*, can act on arrested complex
TFIIF	Eukaryotes, yeast	Increase elongation	Promotes read through of some blocks to elongation, GTF
TTF-1	Eukaryotes	RNAPI termination	Site specific DNA binding protein, blocks RNAPI transcription

containing SWI/SNF promotes elongation downstream of the *Drosophila hsp70* promoter by remodeling nucleosomes downstream of the promoter (14). The GTFs and purified human RNAPII can form preinitiation complexes and initiate transcription on a promoter-proximal chromatin-remodeled template but cannot undergo productive transcription elongation. A novel factor termed FACT (facilitates chromatin transcription) then was purified and identified as a heterodimeric complex composed of the human homolog of the *S. cerevisiae* Spt16 and the HMG-1-like protein SSRP1. The FACT is not ATP-dependent and is thought to function as a histone chaperone (112,113).

Several factors were identified for their ability to increase the catalytic rate and processivity of the RNAP. NusG is a factor from *E.coli* that stimulates escape from pausing at class II (hairpin-less) pause sites by a mechanism that inhibits backtracking (5). The eukaryotic factors that increase the rate of elongation include TFIIF, Elongin, and ELL. The general transcription factor TFIIF is not only required for transcription initiation but remains associated with the polymerase for stimulation of elongation and read through of some blocks to elongation (67,153). TFIIF is phosphorylated by TFIIH and P-TEFb, although the functional significance of this phosphorylation is not known (43,120). In addition, TFIIF also was shown to partially inhibit Factor 2, a termination factor important for the release of early elongation complexes (117).

Elongin and ELL are both implicated in oncogenesis. Elongin is a heterotrimeric complex of A, B, and C subunits. Elongin A is the catalytic subunit and capable of *in vitro* elongation stimulatory activity which is stimulated by association with Elongin B and C (6,47,152). The Elongin BC complex also can interact with the product of the von Hippel-Lindau (VHL) tumor suppressor gene (68). Mutation of the VHL gene

predisposes affected individuals to a variety of cancers including clear-cell renal carcinoma, multiple endocrine neoplasias, and renal hemangiomas (77). A vast number of naturally occurring VHL mutations show reduced binding to the Elongin BC complex (68). It was thought initially that the binding of Elongin BC to VHL and Elongin A represented two independent and mutually exclusive events. However, recent data indicate that Elongin BC is 100- to 1000-fold more abundant than Elongin A and VHL in cell extract (31).

The product of the human *ELL* gene stimulates the rate of elongation by RNAPII by suppressing transient pausing of the polymerase at many sites along the DNA template. This stimulation occurs using purified core RNAPII on a promoter-less template, indicating that stimulation occurs through interactions with RNAPII, the template DNA, or the nascent transcript (63,143). The ELL factor also is capable of inhibiting transcription initiation by binding RNAPII and by preventing its entry into the preinitiation complex (143). Acute myeloid leukemia is associated with translocations of the human *ELL* gene and the *MLL* gene. It is unknown how this fusion protein results in acute myeloid leukemia. The ELL factor recently was purified as a complex with three other proteins which was termed the "Holo-ELL" complex (142). Unlike ELL, however, Holo-ELL does not negatively regulate the polymerase in transcription initiation. A model was proposed where one of the associated proteins in the Holo-ELL complex regulates the transcription inhibitory activity of ELL and deletion of this domain (such as in the MLL-ELL translocation) overrides this regulation (142).

The current model of transcription elongation proposes that the protein-DNA contacts downstream of the RNAP are responsible for the stability of the elongation

complex. The model also proposes that closing of the RNAP around the DNA locks the enzyme into a transcriptionally processive conformation (140). This complex responds to both DNA and RNA sequences that may open the transcription complex and slow the rate of nucleotide addition. The role of some elongation factors may be to stabilize the polymerase in the closed conformation, creating a pause- or arrest-resistant enzyme that is therefore more processive.

Transcription Termination

Termination signals cause the release of RNA and DNA, as well as the RNAP, and can be regulated both positively and negatively. There are three types of prokaryotic transcription termination signals: intrinsic or ρ-independent terminators, ρ-dependent terminators, and persistent RNA-DNA hybrid terminators. Intrinsic terminators require stable RNA hairpin formation followed by 7- to 9-nt of U residues and are independent of extrinsic factors. Pause and termination hairpins probably affect the transcription complex in different ways. Neither the *his* nor the *trp* operon pause RNA hairpin extends to within 10-nt of the transcript 3'-end. The ρ-independent terminator hairpin reaches to within 7- to 9-nt of the RNA 3'-end. This suggests that a termination hairpin may destabilize the ternary complex by disruption of key contacts in the complex that are unaffected by pause hairpin formation, perhaps within the RNA-DNA hybrid (22). The model of intrinsic termination proposes that the U-rich sequence induces a pause that allows time for the formation of the termination hairpin. The hairpin interacts with the RNAP inducing the open, less stable conformation and ultimately results in release of the nascent transcript (155). Rho-dependent termination requires Rho, an RNA-binding protein that possesses both ATPase and RNA-DNA helicase activities. Rho loads onto

the nascent transcript in an unstructured region of the RNA upstream of the terminator and translocates along the nascent RNA. When Rho "catches up with" the paused transcription complex at a termination site, it induces the release of the RNA polymerase and the nascent transcript by destabilizing the RNA-DNA hybrid (11,102,163).

In prokaryotes, most steady state transcript 3'-ends are formed by genuine termination events. In eukaryotes, on the other hand, almost all of the steady state RNAPII transcript 3'-ends are generated by processing of the primary transcript and not by termination of transcription. The processing events are a result of cleavage and polyadenylation sequences within the nascent RNA. Termination of the ternary complex, however, occurs downstream by an unknown mechanism generating heterogeneous transcripts. The transcripts for several RNAPII genes were analyzed by nuclear run-off analysis. The length of these transcripts was shown to range from 100- to 4000-nt downstream of the polyadenylation site (29,51,121). The stability of the elongation complex as demonstrated by its resistance to challenge with sarkosyl or high concentrations of salt, provides a significant question as to the mechanism of termination. There must be some extrinsic signal to induce the extremely stable ternary elongation complex to cease RNA synthesis and terminate. The mechanism of eukaryotic transcription termination may parallel post-replicative transcription in vaccinia virus. Vaccinia transcripts are heterogeneous in length and are not generated by cleavage and polyadenylation. A further discussion of vaccinia virus transcription is found below.

Several transcription termination factors have been identified in eukaryotes. Termination by RNAPI uses a two-component system. One protein binds the DNA at a specific sequence and serves as a block to the elongating polymerase, while the other

protein dissociates the stalled complex. In mice, TTF-1 (Transcription Termination Factor for Pol I) blocks the elongating RNAP and PTRF (Pol I Transcript Release Factor) is responsible for termination of the complex (62,91,123). In yeast, Reb1p is a DNA binding protein that blocks RNAPI. An unidentified element is responsible for induction of termination (123). A second mechanism of eukaryotic termination is proposed to be a result of ATP hydrolysis by DNA and/or RNA polymerase binding proteins. Factor 2, identified in *Drosophila* and humans, is a DNA-dependent ATPase that acts on early RNAPII complexes to induce transcription termination. This activity is counterbalanced by positive acting factors such as P-TEFb (84,165). Transcription termination of vaccinia virus early genes is accomplished by two viral factors, VTF/CE and NPH-I. The early termination signal may be recognized by VTF/CE and termination induced by the DNA-dependent ATPase NPH-I (41,42).

Transcription Antitermination

Lambda phage is most extensively studied for its regulation of elongation and termination. Work by Jeff Roberts on λ phage demonstrated the first example of anti-termination in the positive control of transcription elongation (127). Two proteins are involved in λ anti-termination, λN and λQ, and they function in different ways. Synthesis of a hairpin structure in the nascent transcript recruits N protein. The binding of N to the RNA is stabilized by the assembly of the Nus proteins from *E. coli*. The binding of N to the RNA 5'-end is transmitted to the ternary complex through interaction of N with the RNAP. The N protein becomes a stable polymerase subunit during transcription and the nascent transcript loops. The result is stimulation of transcription elongation and transcription through pause and arrest sites (38,155). The Q protein, on

the other hand, is a DNA binding protein that interacts with RNAP paused at a specific site near the promoter. This results in a transcription complex that can read through downstream termination signals. Both N and Q regulated read through allows the phage to complete the lytic portion of its life cycle.

Vaccinia Virus Biology

Vaccinia virus historically served as a superb model for transcription. The prototypic Orthopoxvirus has a linear double-stranded DNA genome of 192,000 base pairs, which it replicates in the cytoplasm of the infected host cell. Because of the cytoplasmic site of infection, the virus encodes most of the enzymatic machinery necessary for both viral RNA and DNA metabolism. Many of the viral-encoded enzymes have structural and functional similarities to the host cell enzymes. The viral RNA polymerase is eukaryotic-like, composed of 2 large subunits (RPO147 and RPO132) with approximately 30% identity to the Rpb1 and Rpb2 subunits of *S. cerevisiae* and 6 small subunits, one of which (RPO7) is homologous to Rpb12 (Fig. 1) (98). In addition, the viral RPO30 subunit shares 23% amino acid identity and is structurally homologous to mouse SII, the mammalian transcription elongation and cleavage stimulatory factor (1). During infection, viral genes are expressed in a transcriptional cascade encompassing three stages as follows: early, intermediate, and late. Each stage requires trans-acting factors for transcription initiation that are synthesized in the previous stage thus providing the basis for sequential regulation. Biochemical and biological experiments during the past few years showed that elongation and termination of all three transcriptional stages are also regulated events.

The vaccinia virion is composed of a biconcave core containing the viral genome surrounded by a lipid bilayer. Infection of the host cell involves membrane fusion and internalization of the core (Fig. 4). The transcriptional cascade commences with early mRNA synthesis using the enzymes and factors present within the core. The early mRNA is extruded into the cytoplasm and translated on host cell polysomes. These early mRNAs encode the proteins required for DNA replication, the RNA polymerase, and the intermediate transcription factors. Early mRNA synthesis is followed by DNA replication. Intermediate gene transcription then generates the transcription factors necessary for late mRNA synthesis. Late proteins include the early transcription factors to be packaged within the virion for the next round of infection, as well as the structural proteins (98).

Each of the three gene classes is regulated by its own set of cis-acting elements. This is the framework for regulating the timing of gene expression. The specific and distinct critical sequences required for initiation of stage-specific transcription are essential for recognition by different trans-acting factors (99). These factors are discussed below. Early, intermediate, and late-stage promoter sequences are each approximately 30-bp in length, divided into core and initiator regions that were defined by saturation mutagenesis. The initiator region includes the site of transcription initiation, designated as +1. The core region is approximately 15-bp in length and is located from –15 to –30. The intervening DNA, between the core and initiator regions, is defined as the spacer region and is insensitive to mutation. The stringency of the critical

Fig. 4. **Vaccinia virus life cycle.** Vaccinia virus enters the host cell and undergoes early transcription, followed by DNA replication, intermediate transcription, and late transcription. Virions are assembled, packaged, and released for the next round of infection. This figure was a generous gift from Richard C. Condit.

IMV membrane
lateral body
core
IMV
uncoating I
uncoating II
early mRNA
early proteins (VITF)
DNA replication
intermediate mRNA
intermediate proteins (VLTF)
late mRNA
late proteins (VETF)
nucleus
IMV
IEV
EEV
EEV membrane

sequence regions differs among the three classes. Early promoters contain a conserved critical core region, from nucleotides -13 to -27, and a less stringent initiator region where the only requirement is a purine at +1. The intermediate promoter core region resembles that of early promoters in A+T-richness but differs in specific sequence. The core region spans from nucleotides -14 to -24. The initiator region differs from early promoters and is defined by a tetranucleotide sequence (TAAA) that is more similar to late initiator regions. Late promoters have a less stringent A+T-rich core region spanning from nucleotides -10 to -15 separated by a 6-bp spacer region from the TAAAT initiator (96,97). Vaccinia virus transcription apparently does not require enhancer elements, the sequences found upstream of eukaryotic promoters that are important for activated transcription initiation. Most viruses do not respond to environmental signals and therefore do not evolve an unnecessary level of sophisticated regulation. The vaccinia genome also differs from eukaryotic genomes by the absence of chromatin packaging although there may be viral proteins bound to the DNA.

In addition to the differences in promoter sequence, the three gene classes also differ in the formation of the transcript 5'- and 3'-ends. Early mRNAs usually contain a short 5'-untranslated region that is capped, but otherwise unmodified. Termination occurs downstream of a sequence specific termination signal producing early mRNA of discrete length that is 3'-polyadenylated (98). Intermediate and late mRNAs initiate within the AAA element of their core promoters but the resulting RNAs contain additional 5'-A residues incorporated by slippage of the polymerase. This results in a "poly(A) head" that is 30- to 50-nt in length and capped (2,116,139). At intermediate and late times during infection the RNA polymerase does not recognize the early termination

signal and synthesizes 3'-heterogeneous transcripts that are polyadenylated (98). This implies that the mechanism for post-replicative gene 3'-end formation is different from termination of early genes.

Vaccinia Virus Early Gene Transcription

Early transcription differs from the post-replicative stages of transcription in that it occurs mainly in the virion, as opposed to the infected cell cytoplasm. An *in vitro* early transcription system was developed using purified viral transcription factors isolated from the virion (48,98). Early gene transcription requires the vaccinia early transcription factor (VETF), the RNA polymerase-associated protein RAP94, (the product of the *H4L* gene), and the viral RNA polymerase for promoter-specific initiation (Table 2) (16,170). VETF binds the core region of the early promoter, as well as DNA downstream of the RNA start site, and alters the conformation of the DNA template. The DNA-dependent ATPase of VETF is not required for promoter binding but is essential for transcription. The ATPase activity may be a requirement for promoter clearance (15,18,83). It is important to emphasize the requirement for a vaccinia RNA polymerase that contains the RAP94 subunit. There are clearly two forms of the RNAP present in infected cells. The RNAP-RAP94 complex is necessary for initiation at early vaccinia promoters and may allow the polymerase to carry a "memory" of the class of the initiating promoter. Intermediate and late promoters recruit RNAP molecules lacking RAP94. The importance of this subunit will be more apparent in the discussion of transcription termination of the three classes of transcripts. A stable ternary complex is formed after the synthesis of a 7- to 9-nt transcript similar to the prokaryotic polymerase complex. The 5'-cap is synthesized by the time the nascent RNA is 31-nt long, although

Table 2: Vaccinia Virus Transcription Factors

Common Name	Vaccinia Gene	Transcription Stage	Properties/Activity
RNA Pol		All	Multisubunit RNA polymerase
RPO147	J6R		-Homologous to Rpb1
RPO132	A24R		-Homologous to Rpb2
RPO35	A29L		
RPO30/VITF-1	E4L		-Intermediate initiation factor, homologous to Euk. TFIIS
RPO22	J4R		
RPO19	A5R		
RPO18	D7R		
RPO7	G5.5R		-homologous to Rpb12?
RAP94	H4L	Early	Early promoter specificity factor
VETF	A7L D6R	Early	Early promoter binding, DNA-dependent ATPase, Early initiation factor
CE/VTF	D1R D12L	All/Early	Early, intermediate, and late capping enzyme, Early termination factor, Intermediate initiation factor
NPH-I	D11L	Early	DNA-dependent ATPase, RNA helicase, Early termination factor
VITF-2	Cellular	Intermediate	Intermediate initiation factor
YY1	Cellular	Intermediate	Binds intermediate promoters
VLTF-1	G8R	Late	Late initiation factor
VLTF-2	A1L	Late	Late initiation factor
VLTF-3	A2L	Late	Late initiation factor
VLTF-4	H5R	Late	Late transactivator
VLTF-X	Cellular	Late	Late initiation factor
	A18R	All	DNA helicase, DNA-dependent ATPase, Early, intermediate, and late transcript release factor
	G2R	Intermediate and Late	Intermediate and late elongation factor
Poly(A) Pol		All	Poly(A) polymerase (PAP)
	J3R		-PAP stimulatory subunit, 2'O-methyl transferase, Intermediate and late elongation factor
	E1L		-PAP catalytic subunit

stable association of the capping enzyme with the complex does not occur until the nascent transcript is 51-nt in length (52).

Early gene mRNA 3'-ends are formed by termination and not endonucleolytic cleavage (130,145). The newest model for early termination evolved from recently published data demonstrating a protein-protein interaction between RAP94 and NPH-I (94). Since only RNA polymerase containing RAP94 is capable of initiating transcription from early promoters, the specificity of the early transcription termination system may be explained by the physical interaction between RAP94 and NPH-I. The interaction suggests that RAP94 functions as a transcription termination cofactor, recruiting NPH-I to the transcription complex (94). In the absence of RAP94, as in intermediate and late transcription complexes, NPH-I is not recruited to the ternary complex and recognition of the termination signal does not occur. NPH-I requires single-stranded DNA to activate its ATPase activity and the ATPase activity is necessary for termination. The most obvious source of single-stranded DNA in the transcription complex is the nontemplate strand in the transcription bubble. The model proposes that the vaccinia termination factor (CE/VTF) is poised to scan the RNA for the termination signal, UUUUUNU. Recognition of the U_5NU signal by CE/VTF may induce conformational changes to make the single-stranded DNA available to NPH-I. The activation of the ATPase activity of NPH-I results in termination and release of the nascent transcript 20- to 50-nt downstream from the termination signal (28,42,94).

Vaccinia Virus Intermediate Gene Transcription

The intermediate stage of vaccinia gene transcription can be reconstituted in vitro by the use of hydroxyurea-treated infected cell extracts. Several proteins were shown to

be required for intermediate transcription initiation although initiation has not been reconstituted from purified factors. These include the RNA polymerase (-RAP94), capping enzyme (CE/VTF), VITF-1 (E4L/RPO30), an unidentified cellular factor found in the nucleus of uninfected HeLa cells and distributed between the cytoplasm and the nucleus of infected cells (VITF-2), and a two-subunit enzyme, VITF-3, composed of the protein products from open reading frames *A8R* and *A23R* (Table 2) (1,131-134). The use of a cellular factor, VITF-2, for intermediate initiation may be a regulatory mechanism between the early and post-replicative stages of the virus life cycle by indicating whether a cell has been activated for optimal replication (98). It is hypothesized that VITF-3 also could be responsible for regulation of post-replicative gene transcription. The VITF-3 subunits are synthesized from early genes and the mRNAs are not detected after 6 hours post infection. Therefore the regulation could be due to a cessation of synthesis of these viral transcripts or competition of more abundant late transcription factors (134). The cellular transcription factor, YY1, is the first cellular factor identified for its role in vaccinia transcription. YY1 was thought to bind the late gene promoter I1L but recent evidence indicates that the I1L promoter belongs to the intermediate class (Steven Broyles, personal communication) (17). The YY1 protein activates transcription from the intermediate protein *in vitro* and requires its DNA binding domain (17). The intermediate RNA polymerase complex does not recognize early termination signals and synthesizes a heterogeneous family of intermediate transcripts that differ at the 3'-end (98). This implies that if there are cis-acting termination sequences in the DNA they are likely to be ubiquitous and/or highly degenerate.

Vaccinia Virus Late Gene Transcription

Late stage vaccinia mRNA transcription is reconstituted *in vitro* by the use of infected cell cytoplasmic extract. Several factors required for late gene transcription initiation were identified, however, additional factors are still being sought as transcription cannot be reproduced from purified factors alone (Table 2). Three intermediate proteins encoded by the open reading frames of *A1L* (VLTF-2), *A2L* (VLTF-3), and *G8R* (VLTF-1), are necessary for late gene transcription initiation (64,65,160,171). Both A1 and A2 are zinc binding proteins (65) and G8 interacts with itself and A1, as demonstrated by the yeast two-hybrid system (92). An additional viral factor, VLTF-4 encoded by the *H5R* open reading frame, is synthesized early and late during infection and stimulates late gene transcription (70,71). A cellular factor, VLTF-X, was also described as necessary for *in vitro* transcription of late genes and is an RNA binding protein (Cynthia Wright, personal communication) (50,159). Similar to intermediate transcription, the late transcription complex does not recognize early termination signals that are frequently present within the coding region of late genes and generates long transcripts with heterogeneous 3'-ends (98).

Identification and Characterization of Vaccinia Virus Transcription Elongation and Termination Factors

The power of vaccinia virus lies in the ability to genetically manipulate the genome and study gene expression *in vivo*. During the 1970s and 1980s several groups isolated several collections of vaccinia virus temperature-sensitive mutants. Genetic characterization of these mutants revealed some mutants that have noticeable effects on the transcript 3'-ends. Two notable complementation groups are represented by the *G2R*

and *A18R* mutants. These mutant viruses were chosen for further study of vaccinia virus transcription elongation and termination.

The A18 Protein

Cts23, a temperature sensitive virus containing a mutation in the gene *A18R*, shows an abortive late phenotype. Viruses designated as abortive late show a defect in protein synthesis and do not produce progeny virions under the nonpermissive conditions. At the nonpermissive temperature the *A18R* mutant viruses show a drastic decrease in the level of late steady state RNA (8). Transcriptional analysis of several vaccinia genes using northern blots, RNase protection, and RT-PCR analysis determined that mutations in the gene *A18R* result in readthrough transcription from intermediate promoters into downstream genes (115,163). These transcripts are longer than those in a Wt infection. The vaccinia genome contains open-reading frames that are transcribed in both rightward and leftward directions. Therefore, in some regions of the genome readthrough transcription results in the synthesis of complementary strands of RNA. The elevated levels of double-stranded RNA induce the cellular 2'-5'A pathway resulting in the degradation of late viral messages and accounts for the abortive late phenotype (8). The vaccinia *A18R* gene encodes a 56-kDa protein that is expressed throughout infection and packaged in virions (146). The A18 protein is both a 3'-5' DNA helicase and a DNA-dependent ATPase (9,147). Based on the phenotypic analysis of Cts23 and the data presented in this dissertation, the A18 protein is a transcript release factor and possibly a transcription termination factor.

The treatment of Wt virus with the anti-poxviral drug isatin-β-thiosemicarbazone (IBT), results in the synthesis of longer than Wt transcripts at intermediate and late times

during infection, similar to the effect of the *A18R* gene mutation. This implies that IBT promotes readthrough transcription in a Wt virus. The exact mechanism of IBT action is not known. We hypothesize that the target of IBT is involved in transcription termination. This is supported by the isolation of IBT-dependent mutants that have phenotypes in post-replicative transcription elongation and termination.

The G2 Protein

The *in vivo* phenotypic analysis of the *G2R* gene was enabled with the use of two conditional lethal mutants: Cts56 and G2A. Cts56 is a temperature-sensitive mutant that requires the anti-poxviral drug IBT for growth at the non-permissive temperature (40°C) and is IBT-resistant at the permissive temperature (31°C). G2A is an IBT-dependent deletion mutant that plaques only in the presence of IBT at 37°C (93). The *G2R* mutants appear to have normal initiation of all three gene classes and early mRNA structure is unaffected. However, intermediate and late mRNAs are reduced in size as a result of truncation from the 3'-end, suggesting an effect on transcription elongation (11). The *G2R* gene is expressed early and predicted to encode a 26-kDa protein.

The G2A mutant virus is not only IBT-dependent but is also an extragenic suppressor of *A18R* mutants. Theoretically, the reduced elongation seen in a *G2R* mutant virus is compensated by the readthrough transcription that results from an *A18R* mutation or IBT treatment. Alternatively, the enhanced elongation in *A18R* mutants and IBT treatment is compensated by a mutation in the *G2R* gene (35).

The viral *H5R* gene product was shown to associate directly with the G2 protein (12). The H5 protein is an abundant phosphoprotein found associated with virosomes (10), and it was shown to stimulate late viral transcription *in vitro* (70). We believe the

G2 protein functions in a Wt infection by enhancing transcription elongation at intermediate and late times during infection. Together, the evidence suggests that the A18, G2, and H5 proteins are all associated either directly or indirectly as a complex *in vivo* (12).

The J3 Protein

The J3 protein was previously characterized as a bifunctional (nucleoside-2'-O-)-methyltransferase and as a processivity factor for the heterodimeric viral poly(A) polymerase (138). This 39-kDa protein is expressed throughout infection and packaged in virions (100,103). Isolation of a *J3R* mutation as an extragenic suppressor of the *A18R* mutation has led to the hypothesis that J3, like G2, functions as a positive transcription elongation factor. In fact, several *J3R* mutations were isolated by selecting for IBT-dependent mutants, all of which were null mutations that synthesize no detectable J3 protein (78). Northern blot and structural analysis of the *F17R* gene indicate that *J3R* mutant viruses produce intermediate and late transcripts that are specifically 3'-end truncated consistent with the reduction in large proteins late during infection. Analysis of two *J3R* mutant viruses, which retain or lack the poly(A)-stimulatory activity, demonstrate that the poly(A)-stimulatory activity of J3 is separable from the elongation activity (78,162).

Summary

The goal of this dissertation is to provide a biochemical characterization of the regulation of vaccinia virus transcription elongation and termination. The *in vivo* analysis of several vaccinia virus mutants in the genes *A18R*, *G2R*, and *J3R*, provided the initiative for our hypothesis. We propose that these three viral proteins, in conjunction

with other viral or cellular factors, regulate vaccinia transcription elongation and termination at post-replicative times during infection. These proteins work as positive and negative factors to balance the synthesis of mRNA of the correct length. We further propose, based on recent data from studies of vaccinia early transcription termination, that the vaccinia transcription machinery may exist as a holoenzyme, similar to those demonstrated for both prokaryotic and eukaryotic systems. This holoenzyme is composed of the factors necessary for processing of mRNA 5'- and 3'-ends in addition to the machinery for transcription initiation, elongation, and termination. This hypothesis is supported by the physical recycling of viral proteins for different functions during the transcription cascade. For example, the viral capping enzyme is involved in 5'-cap formation of all 3 stages of transcription, as well as serving as an early termination factor and an intermediate initiation factor. The J3 protein is another example of a recycled protein, as it has activity both in 5'-cap formation and 3'-end polyadenylation, as well as elongation. There may be two forms of this holoenzyme in the cell, as early promoters clearly are selective in recruiting RNA polymerase molecules containing RAP94, whereas intermediate and late promoters recruit the RAP94(-) polymerase. The data presented in this dissertation demonstrates that at least one of these proteins, A18, is directly involved in post-replicative transcription termination. We have also identified a cellular factor that appears to participate in transcription termination.

CHAPTER 2
MATERIALS AND METHODS

Eukaryotic Cells, Viruses, and Bacterial Hosts

A549 cells, wild type vaccinia strain WR, and *A18R* temperature-sensitive mutant Cts23, and the conditions for their growth, infection, and plaque assay have been described previously (32-34). *Escherichia coli* DE3 pLysS contains an isopropyl-1-thio-β-B-inducible chromosomal copy of the bacteriophage T7 RNA polymerase gene (149).

Plasmids

All plasmids used for transcription are based on pC_2AT19 (135) containing a 375-nt G-less cassette cloned into pUC13 with the total size approximately 3-kb. pG8G, pVGFG, and pCFW10 contain upstream of the 375-nt G-less cassette promoters from the intermediate vaccinia gene *G8R*, the early vaccinia gene *C11R*, and the late vaccinia gene *F17R* (32,161), respectively. pSB24 contains a synthetic early promoter upstream from the 375-nt G-less cassette (85). pG8GX is a derivative of pG8G that contains the vaccinia gene *G8R* intermediate promoter upstream of a 3'-truncated, 94-nt G-less cassette derived from pC_2AT19 (76). pSB23term contains a synthetic early promoter upstream of a 540-nt G-less cassette and contains the early termination signal U_5NU (28,32).

pG8GI is a derivative of pG8G that contains the vaccinia gene *G8R* intermediate promoter upstream of a 3'-truncated, 37-nt G-less cassette derived from pC_2AT19. The *G8R* promoter and the 5' 37-nt of the pC_2AT19 cassette were PCR-amplified from pG8G using an upstream primer that hybridized approximately 270-nt upstream of the *G8R*

promoter flanked with a *Sac*I site and a *Sal*I site and a downstream primer that contained nucleotides 18 to 37 of the G-less cassette flanked with a *Sma*I site and a *Bam*HI site. The PCR-amplified fragment was cleaved with *Sac*I (upstream) and *Bam*HI (downstream) and cloned into the vector pGEM3ZF$^-$, which had also been cleaved with *Sac*I and *Bam*HI. The *Sma*I site at the 3' end of the resulting truncated G-less cassette serves to efficiently arrest transcription of the G-less cassette, and the upstream *Sal*I site was used for identification of the desired clone. Accurate transcription of the pG8GI G-less cassette should yield RNA of approximately 37-nt in length.

pG8G4a and pG8fe are derivatives of pG8GI that contain the vaccinia gene *G8R* intermediate promoter upstream of a 3'-truncated, 37-nt G-less cassette derived from pC_2AT19. The pG8G4a plasmid contains the vaccinia late gene *A10L* which was PCR-amplified from purified Wt vaccinia virus DNA using an upstream primer that hybridized to nucleotides 1 to 22 corresponding to the initiating ATG of *A10L* flanked by a *Sma*I site and a downstream primer that hybridized to the 3' 19-nt of the *A10L* gene flanked by a *Hind*III site. The PCR-amplified fragment was cleaved with *Sma*I and *Hind*III and cloned into pG8GI that had also been cleaved with *Sma*I and *Hind*III. The resulting plasmid contains the 37-nt G-less cassette followed by the coding sequence of the *A10L* gene. The pG8fe plasmid contains the vaccinia late genes *F17R* and *E1L* that were PCR-amplified from purified Wt vaccinia virus DNA using an upstream primer that hybridized to nucleotides 1 to 23 corresponding to the initiating ATG of *F17R* flanked by a *Sma*I site and a downstream primer that hybridized to nucleotides 1 to 20 corresponding to the initiating ATG of *E1L* flanked by a *Pst*I site. The PCR-amplified fragment was cleaved with *Sma*I and *Pst*I and cloned into pG8GI that had also been cleaved with *Sma*I and *Pst*I.

The resulting plasmid contains the 37-nt G-less cassette upstream of the coding sequence of the *F17R* and *E1L* genes.

p16A18 (9) contains the vaccinia virus gene *A18R* coding sequence inserted in frame downstream from an amino-terminal polyhistidine tag in the vector pET16b (Novagen).

Infected Cell Extracts for Transcription

Confluent 100-mm dishes of A549 cells were either mock-infected or infected with vaccinia virus with a multiplicity of infection of 15 and incubated at 40°C for 16 h in the presence of 10 mM hydroxyurea or in the absence of drug. Extracts were prepared as described (32). Briefly, vaccinia-infected cell monolayers were permeabilized with lysolecithin, harvested, treated with micrococcal nuclease, clarified by centrifugation, and stored at -70°C. Total protein concentration was determined by the Bradford protein assay (Bio-Rad).

Immobilized DNA Templates

All templates used for transcription were immobilized by binding linearized plasmid DNA to paramagnetic beads. One set of immobilized templates, including NpG84a, NpG8G, NpG8fe, NpSB24, and NpCFW10, were generated by linearization with *Nde*I, which cleaves the DNA template 220-nt upstream from the promoter. The resulting templates contain a 375-nt G-less cassette and approximately 2400-nt DNA downstream from the G-less cassette. Two additional shorter templates, N/VpG8G and N/VpG8GX, were constructed by restriction digest with *Nde*I and *Vsp*I. The resulting templates contain 220-bp DNA upstream from the *G8R* intermediate promoter and either 540- or 260-bp downstream for transcription (Fig. 8B). In all cases the cleaved DNA fragments were end-filled with Klenow, dCTP, dGTP, and dATP, and biotin-16-dUTP

(Roche Molecular Biochemicals). The biotinylated DNA was separated from the free nucleotides using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). The DNA was eluted from the column in 100 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and adjusted to 1 M NaCl. DNA samples were then incubated with streptavidin-conjugated Dynabeads M280 (Dynal) in 1 M NaCl/TE for 30 min at 42°C to generate bead-bound templates. Beads with bound DNA were concentrated using a magnet and washed twice in 1 M NaCl/TE, followed by two washes in TE. The bead-bound DNA was stored in TE at 4°C.

In Vitro Transcript Release Assay

The purpose of this dissertation was to develop an *in vitro* system to characterize the A18, G2 and/or J3 proteins. The reaction described here represents the final conditions of this assay as used to measure transcript release. Variations on this assay were used during the development and are described in the text of Chapter 3.

Transcription reactions were performed in three phases, initiation, pulse, and chase. Reactions (25 µl) contained a final concentration of 25 mM HEPES, pH 7.4, 4.5% glycerol, 80 mM KOAc, 5 mM $MgCl_2$, 1.6 mM DTT, 1 mM ATP, 5 µl of bead-bound DNA template, and 15 µl of extract from hydroxyurea-treated wild type vaccinia-infected cells. Reactions were incubated at 30°C for 10 min to form initiation complexes. The pulse phase was initiated by adding 3 µl of a solution containing 11 mM ATP, 11 mM GTP, 6 mM UTP, and 6 µCi of [α-P^{32}] CTP (~3000 Ci/mmol stock) such that the final concentration is 2.1 mM ATP, 1.1 mM GTP, 0.6 mM UTP, 22.3 mM HEPES, pH 7.4, 4% glycerol, 71.4 mM KOAc, 4.5 mM $MgCl_2$, and 1.4 mM DTT in a total of 28 µl. These reactions were then incubated at 30°C for 30 s. The reactions were stopped by

placing the tube on a magnet on ice. The pellets were washed with 1 to 1.5 pulse reaction volumes of high salt transcription buffer (5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 1.6 mM DTT, 1 M KOAc, and 7.5% glycerol), followed by three washes in 1 to 1.5 pulse reaction volumes of low salt transcription buffer (5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 1.6 mM DTT, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol). The chase phase was done by adding to the resuspended complexes a mixture of NTPs, extract, and proteins in a final volume of 25 μl containing 25 mM HEPES, pH 7.4, 4.5% glycerol, 80 mM KOAc, 5 mM $MgCl_2$, 1.6 mM DTT, 600 μM ATP, 600 μM GTP or 10 μM 3'-OMeGTP, 600 μM UTP, 1.2 mM CTP, 20 units RNasin, and purified protein or extract as indicated. Chase reactions were performed at 30°C for various times. The beads were concentrated using a magnet, and the 25 μl supernatant was removed to a separate tube. One hundred seventy five microliters of "PK mix" (114 mM Tris-HCl, pH 7.5, 14 mM EDTA, 150 mM NaCl, 1.14% SDS, 40 μg of glycogen, 230 μg/ml proteinase K) was added, and reactions were incubated at 37°C for 30 min. Reactions were extracted once with 175 μl of phenol/chloroform. Nucleic acids were precipitated by addition of 50 μl 10 M ammonium acetate and 150 μl isopropyl alcohol, incubation at room temperature for 30 min, and centrifugation for 20 min. Pellets were washed once with 70% ethanol, dried, and resuspended in 10 μl of formamide loading buffer. Samples were denatured at 90°C for 3 min and loaded on a 6% 8 M urea-PAGE. Gels were fixed, dried, and analyzed by autoradiography and phosphorimagery. Released transcripts were expressed as a percentage derived by dividing the quantity of transcripts in the supernatant by the total quantity of transcripts in both the supernatant and associated with the beads.

Induction and Preparation of Extract from *E. coli*

An overnight culture of pLysS cells harboring the p16A18 plasmid was used to inoculate 1 liter of L-broth, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was incubated at 37°C to an A_{600} of 0.5. Isopropyl-1-thio-β-D-Galactopyranoside was added to a final concentration of 1 mM, and the culture was incubated at 37°C for 4 h. The cells were pelleted and stored at –70°C overnight. All subsequent procedures were performed at 4°C. The thawed bacterial pellet was resuspended in 50-ml of lysis buffer (50mM Tris, pH 7.5, 0.15M NaCl, 10% sucrose) plus a final concentration of 50 μg/ml lysozyme and 0.1% Triton X-100. The cells were sonicated at 4°C for eight sequences consisting of 15 s on and 45 s off. Insoluble material was removed by centrifugation for 30 min at 18,000 rpm in a Sorvall SS34 rotor at 4°C. For purification of the soluble A18R protein, the supernatant was then chromatographed on a His-Bind (Novagen) column and phosphocellulose column as described below.

His-bind Column and Phosphocellulose Column

The supernatant was mixed for 1 h with 2-ml of nickel-nitrilotriacetic acid-agarose resin (Quiagen) that was equilibrated with lysis buffer. The slurries were poured into a column and washed sequentially with 20-ml of lysis buffer, 20-ml of binding buffer (5mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol), and 20-ml of wash buffer 1 (60 mM imidazole, 0,5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol). Bound proteins were eluted with 20-ml of wash buffer 2 (200 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol) collecting 1-ml fractions. Peak fractions were identified using the Bradford protein assay (Bio-Rad), pooled, and dialyzed overnight against 1 liter of Buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5

μg/μl leupeptin, and 0.7 μg/μl pepstatinA). The dialysate was applied to a 2-ml column of phosphocellulose that had been equilibrated with Buffer A. The column was washed with 5-ml of Buffer A containing 250 mM NaCl. Bound proteins were eluted with 10-ml of Buffer A containing 500 mM NaCl collecting 0.5-ml fractions. Peak fractions were identified using the Bradford protein assay, pooled and dialyzed overnight against 4 changes, 1 liter each, of a solution containing 40 mM Tris-HCl, pH 8, 20 mM KCl, and 40% glycerol. The enzyme was stored at –20°C. The His-A18R protein preparation was greater than 90% pure as judged by PAGE and displayed DNA-dependent ATPase activity of 10,000 nmol of ATP hydrolyzed per min per μg of protein, equivalent to previously reported preparations (9).

Vaccinia virus J3R protein containing both a polyhistidine- and thioredoxin-tag, was prepared in a fashion similar to His-A18R (162).

Polyhistidine-tagged human factor 2, prepared as described (84), was a gift from Dr. David Price (University of Iowa).

Western Blot Analysis

Samples were separated by electrophoresis on 10% SDS-PAGE. The proteins were transferred to nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, 20% methanol at 4°C overnight. Nitrocellulose filters were incubated with monoclonal anti-A18 primary antibody (1:10,000) (12), and the bound antibody was detected using polyclonal anti-mouse horseradish peroxidase-conjugated antibody (1:5000, Amersham Pharmacia Biotech) and enhanced chemiluminescence. Western blotting reagents (Amersham Pharmacia Biotech) were used as described by the manufacturer.

Preparation of Nuclear and Cytoplasmic Fractions of HeLa Cells

HeLa cells grown in suspension culture to a density of 5 x 10^5 cells per ml (a generous gift from Brian O'Donnell) and were harvested for extraction. All subsequent procedures were performed at 4°C. Cell pellets were resuspended in Buffer A at a ratio of 5-ml of buffer per ml of packed cell pellet. Cells were allowed to swell on ice for 10 min followed by centrifugation at 1000 x g for 10 min. The cell pellets were resuspended in Buffer A at a ratio of 2-ml of buffer per ml of packed cell pellet. The cells were ruptured by Dounce homogenization using a tight-fitting pestle. The lysate was centrifuged at 1000 x g for 15 min to pellet nuclei. The nuclear pellet was saved for extract preparation. The supernatant was centrifuged again at 10,000 x g for 15 min and the resulting supernatant was saved and labeled as cytoplasmic extract (HCE). The nuclear pellet was subjected to additional centrifugation at 25,000 x g for 20 min. The pellet was resuspended in 3-ml Buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) for every 1 x 10^9 cells. The nuclei were triturated by Dounce homogenization using a tight-fitting pestle. The homogenate was mixed using a stir bar for 30 min at 4°C, centrifuged at 25,000 x g for 30 min, and dialyzed overnight against Buffer P (20 mM HEPES, pH 7.9, 10% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF). The dialysate was centrifuged at 25,000 x g for 20 min and the resulting supernatant was stored at -70°C as nuclear extract (HNE).

Chromatography and Fractionation

Crude Fractionation of Wt or Cts23 Extract

Extract from Wt- or Cts23-infected A549 cells was chromatographed on 2-ml columns of phosphocellulose (Whatman) or Q-Sepharose (Amersham Pharmacia

Biotech) equilibrated in Buffer A. All steps were performed at 4°C. Extract was loaded on the column, and the column was washed in 4-ml of Buffer A, and 0.5-ml flow-through fractions were collected. Bound proteins were eluted stepwise with 4-ml each of Buffer A containing 0.25, 0.5, and 1 M NaCl, and 0.5-ml fractions were collected. Peak fractions were identified using the Bradford protein assay, pooled, and dialyzed overnight against Buffer A containing 50 mM NaCl. The fractions were stored at -20°C.

HQ Purification

Cytoplasmic extract from uninfected HeLa spinner cells was chromatographed on Porus 20 HQ (PerSeptive Biosystems) equilibrated in bis-Tris propane, pH 6. The column was run using the BioCAD Perfusion Pump (provided by the Protein Chemistry Core Facility, Biotechnology Program, University of Florida) at room temperature. Extract was loaded on the column, and the column was washed in five column volumes of bis-Tris propane, pH 6, and the flow-through fraction was collected and placed on ice. Bound proteins were eluted using a gradient of bis-Tris propane, pH 6 from 0 M to 0.5 M NaCl followed by a wash with 2 M NaCl, and 1-ml fractions were collected and placed on ice. Peak fractions were identified based on absorbance at 280 nm. The fractions were stored at -70°C.

Hydroxyapatite Purification

The hydroxyapatite purification was performed subsequent to purification over Q-Sepharose. Cytoplasmic extract from uninfected HeLa spinner cells was chromatographed on a 2-ml column of Q-Sepharose equilibrated in Buffer P. All steps were performed at 4°C. Extract was loaded on the column, and the column was washed in 4-ml of Buffer P, and 0.5-ml fractions were collected. Bound proteins were eluted using a continuous gradient of Buffer P from 0 M to 1 M NaCl, and 0.5-ml fractions were

collected. Peak fractions were identified using the Bradford protein assay and the *in vitro* transcript release assay. Q-Sepharose fractions 21 to 35 were pooled and dialyzed against Buffer D (20 mM HEPES, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.7 μg/ μl pepstatin A, 0.1 mM PMSF, 0.5 μg/ μl leupeptin) containing 0.01 M phosphate. The dialyzed fractions were chromatographed on a 1.5-ml hydroxyapatite column equilibrated in 0.01 M Buffer D, washed in 4-ml 0.01 M Buffer D collecting 0.5-ml fractions. Bound protein was eluted using a continuous gradient of Buffer D from 0.01 M to 0.4 M phosphate, and 0.5-ml fractions were collected. Peak fractions were determined using the Bradford protein assay. The fractions were stored at -70°C.

Phosphocellulose Purification

Cytoplasmic extract from uninfected HeLa spinner cells was chromatographed on a 2-ml column of DE-52 equilibrated in Buffer P to remove the majority of nucleic acid prior to fractionation of phosphocellulose. The column was washed in 4-ml Buffer P and bound protein was eluted in Buffer P containing 0.5 M NaCl. Peak fractions were determined using the Bradford protein assay and fractions 15 to 19 were pooled and dialyzed against Buffer P. The dialyzed DE-52 fraction was subjected to gradient fractionation on phosphocellulose from 0 M to 1 M NaCl and 0.5-ml fractions were collected. Peak fractions were determined by Bradford protein assay. The fractions were grouped and dialyzed against Buffer P. The fractions were stored at -70°C.

CHAPTER 3
RESULTS

Objectives and Specific Aims

The overall goal of my research is to provide a biochemical characterization of the regulation of vaccinia virus transcription elongation and/or termination. The *in vivo* analysis of the viruses containing mutations in the genes *G2R* and *J3R* indicates that the transcripts synthesized from intermediate and late genes are 3' truncated as compared to a Wt infection (11,78,162). We therefore hypothesize that G2 and J3 function as positive transcription elongation factors. Through the use of Northern blots, RNase protection, and reverse transcriptase-PCR analysis it was determined that virus containing a temperature sensitive mutation in the gene *A18R* synthesize transcripts that are longer than those synthesized during a Wt infection (163). We therefore hypothesize that A18 functions as a negative transcription elongation factor or a termination factor. The goal was to develop and characterize assays for elongation and termination and to simultaneously screen for activity of any or all of the aforementioned proteins. This was a huge undertaking, as there were no established assays for pausing, pause suppression, or termination for the post-replicative genes of vaccinia virus. Therefore, the assays varied until an *in vitro* phenotype was discovered for A18. The subsequent assays focused on characterizing the A18 protein using the new transcript release assay. Clearly there are other experiments that could be pursued to characterize G2 and J3 using the

knowledge gained from the preliminary elongation assays described in this dissertation and those experiments are discussed further in Chapter 4.

Specific Aim 1: Develop an Assay to Determine the Biochemical Activity of A18, G2, and/or J3

The first aim of this project was to characterize the transcription reaction by testing variables important for elucidating elongation and termination factors in other systems. We developed various transcription assays based on a previously described crude system for the study of vaccinia early, intermediate, and late gene transcription initiation (32). Previous experiments showed that crude extract prepared from cells infected under normal conditions is competent for transcription of early, intermediate, and late gene promoters. Since intermediate and late viral gene expressions are coupled to viral DNA replication, treatment of infected cells with a DNA replication inhibitor such as hydroxyurea permits synthesis of only early gene products, including intermediate transcription factors. Thus extracts prepared from cells infected in the presence of hydroxyurea are competent for transcription of intermediate promoters only (32). For most experiments, we chose to use hydroxyurea-treated, intermediate promoter-specific extract for two reasons. First, the best evidence that the A18, G2, or J3 proteins have elongation factor activity is based on *in vivo* studies of intermediate genes (11,78,162,163). Second, we wished to prepare extract from *A18R* mutant infections under non-permissive conditions while at the same time circumventing undesirable pleiotropic effects of the *A18R* mutation. Readthrough transcription from convergent intermediate promoters during *A18R* mutant infections causes double-stranded RNA accumulation, induction of the cellular 2'-5'A pathway, and ultimately activation of RNase L (146,147,163). Hydroxyurea treatment prevents 2'-5'A pathway activation by

preventing intermediate transcription. Cells infected with *A18R* mutant virus at the non-permissive temperature produce less than 10% of the normal amount of A18 protein due to instability of the mutant protein (146). Thus preparation of extracts from *A18R* mutant-infected cells at the non-permissive temperature provides an A18 protein-deficient extract that is otherwise comparable to extract from cells infected with Wt virus under identical conditions.

Due to the fact that the transcription assay was under development and the experiments are not necessarily presented in chronological order, the exact details of the assay as presented in this dissertation may differ between experiments. The variation in the basic steps of the transcription assay can be summarized through the description of a general formula. The general formula for the transcription assay includes: 1) transcription complex formation, 2) ^{32}P-labeling of the nascent RNA, 3) washing of the isolated complex, 4) elongation during a chase step, and 5) RNA isolation. Transcription complexes are assembled on a paramagnetic bead-bound DNA template in one of two fashions; either during a separate incubation of viral extract with the DNA template or concurrent with the pulse reaction containing the viral extract, DNA template, and nucleotides. In both cases initiation and P^{32}-labeling of the nascent RNA occurs during the pulse reaction. The pulse-labeled ternary complexes are isolated using a magnet and washed in transcription buffer containing either 80 mM KOAc (low salt wash) or 1 M KOAc (high salt wash). Transcription elongation continued during the chase step in which the nucleotide concentration and protein composition are varied. The final step is isolation of the RNA. RNA released from the ternary complex is isolated using a magnet to separate bead and supernatant fractions, representing bound and released RNA,

respectively. Alternatively, the total RNA synthesized during the reaction is isolated by not separating the beads from the supernatant. The specific variations used in each experiment will be described relative to this general formula.

Based on the *in vivo* data regarding mutations in the G2, J3, and A18 proteins, we hypothesized that these proteins were positive and negative elongation factors. To test this hypothesis, intermediate promoter-specific transcription complexes established on bead-bound DNA templates were assayed for different patterns of elongation during a chase step that contained limiting nucleotides and Wt or mutant extract or purified protein. This assay is based on the idea that nucleotide starvation enhances pausing of the RNAP thus allowing us to assay the effect of potential transcription elongation factors at various pause sites *in vitro*. To date, we have not defined the activities of either G2 or J3 using the *in vitro* elongation assay and additional experiments are in progress. Experiments to assess the stability of transcription elongation in this assay were performed with the use of sarkosyl or NaCl during the elongation step. The ternary complexes were fairly resistant to challenge with either agent indicative of the stability of the complex during elongation. During this period of experimentation we discovered that *in vitro* the A18 mutant virus, Cts23, did not induce release of nascent RNA at levels similar to the Wt extract. This discovery resulted in the subsequent specific aims and focused on characterization of the A18 protein using the transcript release assay.

Specific Aim 2: *In Vitro* Analysis of the A18 Phenotype

The second specific aim focuses on the characterization of transcript release and the importance of A18 in that process. Intermediate promoter-specific, pulse-labeled transcription complexes established on bead-bound DNA templates were assayed for

transcript release during an elongation step that contained nucleotides and various proteins. Release was analyzed by comparing transcripts present in the supernatant to transcripts in the bead-bound fraction. Extract from Wt-infected cells, but not mock- or Cts23-infected cells, stimulated transcript release. The presence of A18 protein is an absolute but not the sole requirement for transcript release. We also demonstrate that an additional activity, in combination with A18, is necessary for efficient transcript release.

Specific Aim 3: Characterization of the Cellular Factor

Transcript release is achieved using a combination of extract from Cts23- or mock-infected cells plus purified A18 protein. These data suggest that the additional activity necessary for intermediate transcript release is provided by a cellular factor (CF). With the ultimate goal of identifying the cellular factor in mind, we first tested a known cellular termination factor for activity in the vaccinia *in vitro* system. This protein, Factor 2, did not substitute for A18 or CF in the vaccinia transcript release assay. We therefore continued experiments to identify CF using conventional chromatography. The CF is present in both nuclear and cytoplasmic extracts from uninfected HeLa cells. Cytoplasmic extract fractionated over several chromatography resins demonstrated that CF binds to DEAE, Q-Sepharose or HQ, and hydroxyapatite and does not bind to phosphocellulose.

Specific Aim 4: Characterize A18/CF-Dependent Release from All Vaccinia Promoters

The fourth specific aim of my research was to determine the promoter specificity of A18-dependent transcript release. Transcription complexes were formed on early and late vaccinia promoters and assayed for the ability to induce transcript release in the

absence or presence of A18 protein and CF. The combination of A18 and CF is active in inducing release from all three classes of promoters. In addition, CF also enhances release of transcripts terminated by recognition of the vaccinia early gene-specific termination signal.

Specific Aim 1: Develop an Assay to Determine the Biochemical Activity of A18, G2, and/or J3

Formation of Paused Transcription Complexes

Extract from hydroxyurea-treated vaccinia-infected cells was used to assay elongation from linear bead-bound DNA templates containing a vaccinia intermediate promoter as follows. Transcription complexes were assembled and initiated during a 30-minute pulse reaction containing Wt extract, bead-bound template, [α-^{32}P] CTP, ATP, UTP, and 3'-OMeGTP. The ternary complexes, consisting of the DNA template, the transcription apparatus, and the radiolabeled nascent RNA, were purified from nonspecifically bound proteins and unincorporated nucleotides during two washes in a low salt (80 mM KOAc) transcription buffer. The elongation assay was performed by addition of a "chase" mixture containing NTPs, extract, and proteins as indicated. Total labeled RNA was analyzed on a denaturing polyacrylamide gel.

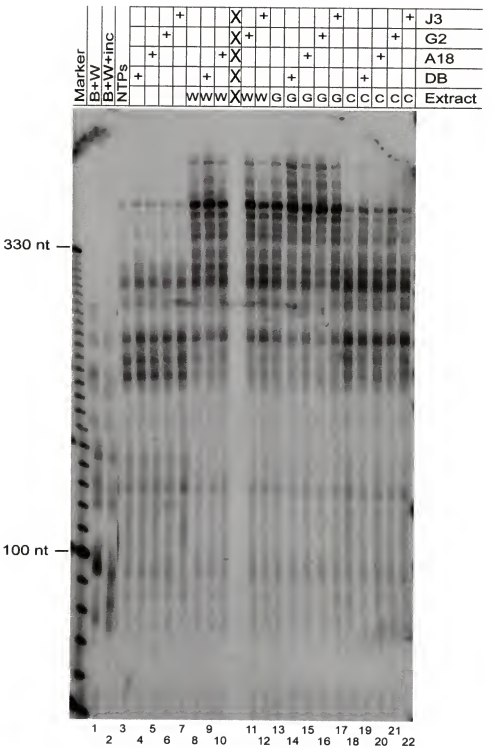
One of the predominant characteristics of identified pause sites is the presence of T-rich sequences in the nontemplate strand (155). To artificially enhance pausing at these sites *in vitro* we used a reduced quantity of UTP in the chase mixture. Transcription was initiated on the NpG84a bead-bound template that contains the late vaccinia gene *A10L* downstream from the vaccinia *G8R* intermediate promoter and a 37-nt G-less cassette. Purified ternary complexes were chased in the presence of 1 mM

ATP, CTP, GTP, and 2 μM UTP to reveal several paused transcription complexes (Fig. 5, Lane 1). We then tested the effect of purified proteins during transcription elongation with reduced UTP concentration (Fig. 5, Lanes 3-5). We detected no change in the elongation pattern due to the presence of A18, G2 or J3 proteins. We also tested the effect of either Wt or mutant extract (G2A or Cts23) in the presence and absence of purified A18, G2, or J3 proteins (Fig. 5, Lanes 6-20). There is an increase in the length of transcripts recovered in the presence of either Wt or G2A extract, although there are still discernible pause bands (Fig. 5, Lanes 6-15). This increase in length of the paused transcripts probably represents the presence of contaminating UTP in the Wt and G2A extracts. There is again no difference with the presence or absence of purified proteins combined with Wt or mutant extract (Fig. 5, compare Lane 6 with Lanes 8-10, Lane 11 with Lanes 13-15, and Lane 16 with Lanes 18-20). We did not titrate the concentration of the proteins used in the assay, so it is possible that an effect would be seen using higher concentrations of purified protein.

Sarkosyl Stability of Elongation and Termination

Sarkosyl is a detergent used to strip factors responsible for elongation from the transcription complex. We tested the effect of sarkosyl on vaccinia virus transcription elongation using a modified *in vitro* transcription protocol. First, transcription complexes were assembled during a separate pre-incubation reaction containing Wt extract and the NpG8fe template (contains the vaccinia *G8R* intermediate promoter, a 37-nt G-less cassette, and the vaccinia *F17R* and *E1L* open reading frames). Transcription was initiated and the nascent transcript radiolabeled with the addition of [α-^{32}P] CTP, ATP, GTP, and UTP during a 30-second pulse reaction. Nonspecifically bound proteins and

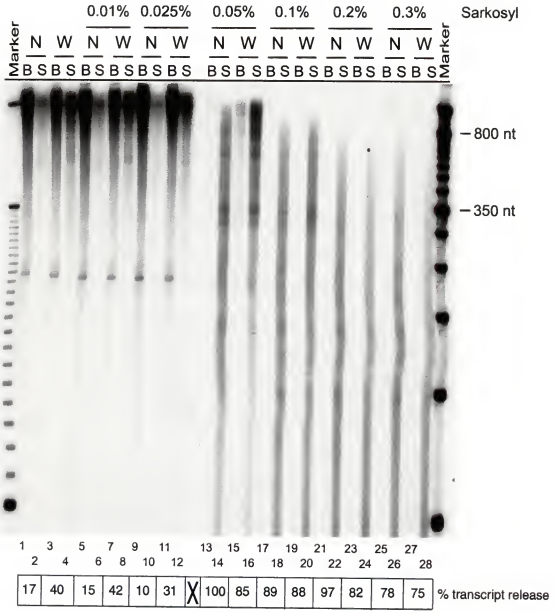
Fig. 5. **Formation of paused ternary complexes.** Figure shows an autoradiogram of an *in vitro* elongation assay. Transcription complexes were assembled and initiated in a mixture containing Wt extract, immobilized NpG8G4a DNA containing the vaccinia *G8R* intermediate promoter, 3.5 µCi [α-^{32}P] CTP (~3000 Ci/mmol stock), 1 mM ATP, 1 mM UTP, 10 µM 3'-OMeGTP for 30 min at 30°C. The labeled complexes were isolated using a magnet and washed three times in low salt transcription buffer (*B+W, lane* 1). Elongation was continued in the absence (*B+W+inc, lane* 2) or presence of 1 mM ATP, CTP, GTP, and 2 µM UTP alone (*NTP, lane* 3) or with additional 3µl protein buffer (*DB*) 75 ng vvHis-A18 (*A18*), 75 ng vvHis-G2 (*G2*), 1 µg J3 (*J3*), 37.5 µg Wt extract (*W*), 18.75 µg G2A extract (*G*), or 7.5 µg Cts23 extract (*C*) as indicated by the (+) for 10 min at 30°C. The transcripts were analyzed by 4% 8 M urea-PAGE. Sizes, in nt, are shown on the *left*.


Marker
B+W
B+W+inc
NTPs
J3
G2
A18
DB
Extract
330 nt
100 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

unincorporated nucleotides were removed from the ternary complex during a single wash in low salt (80 mM KOAc) transcription buffer. The isolated complexes were resuspended in a solution containing nucleotides, Wt extract or buffer, and increasing concentrations of sarkosyl and incubated for 20 minutes. After the elongation reaction the beads were concentrated using a magnet, the supernatant was removed to a separate tube, and the labeled RNA in each fraction was analyzed on a denaturing polyacrylamide gel. Released transcripts were expressed as a percentage derived by dividing the quantity of transcripts in the supernatant by the total quantity of transcripts in both the supernatant and associated with the beads.

In the absence of sarkosyl and Wt extract low levels of transcripts are released into the supernatant (Fig. 6, Lanes 1 and 2). With the addition of Wt extract the amount of transcripts released into the supernatant is increased by at least 2-fold (Fig. 6, compare Lanes 1 and 2 with Lanes 3 and 4). This indicates that there may be additional factors necessary for transcript release provided by Wt extract but not associated with the washed transcription complex. The low level of transcript release in the absence of Wt extract may be a result of the single low salt wash that may not efficiently remove non-specifically bound proteins. There was no effect on Wt extract-dependent transcript release with the addition of sarkosyl from 0.01% to 0.025% (Fig. 6, Lanes 5-12). Concentrations of sarkosyl from 0.05% to 0.3% inhibited transcription elongation and resulted in release of nascent RNA regardless of the presence of Wt extract, indicating that these complexes are not stable to high concentrations of sarkosyl (Fig. 6, Lanes 13-28).

Fig. 6. **Instability of elongation complex to high concentrations of sarkosyl.** Transcription complexes were assembled as described in Fig. 3 on immobilized NpG8fe DNA containing the vaccinia *G8R* intermediate promoter and the *F17R* and *E1L* open reading frames. Isolated ternary complexes were chased in a mixture containing 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*N, lanes* 1 and 2, 5 and 6, 9 and 10, 13 and 14, 17 and 18, 21 and 22, and 25 and 26) or in the presence of 15 μg Wt extract (*W, lanes* 3 and 4, 7 and 8, 11 and 12, 15 and 16, 19 and 20, 23 and 24, and 27 and 28). Increasing concentrations of sarkosyl were used in the presence of only NTPs (*N*) or NTPs and Wt extract (*W*) as follows: 0.01% (*lanes* 5-8), 0.025% (*lanes* 9-12), 0.05% (*lanes* 13-16), 0.1% (*lanes* 17-20), 0.2% (*lanes* 21-24), 0.3% (*lanes* 25-28). Elongation was continued for 20 min at 30°C and the bead-bound RNA (*B*) was separated from released RNA (*S*) using a magnet. The transcripts were analyzed by 6% 8 M urea-PAGE. Percent transcript release is indicated in the table below the autoradiogram. Sizes, in nt, are shown on the *right*.

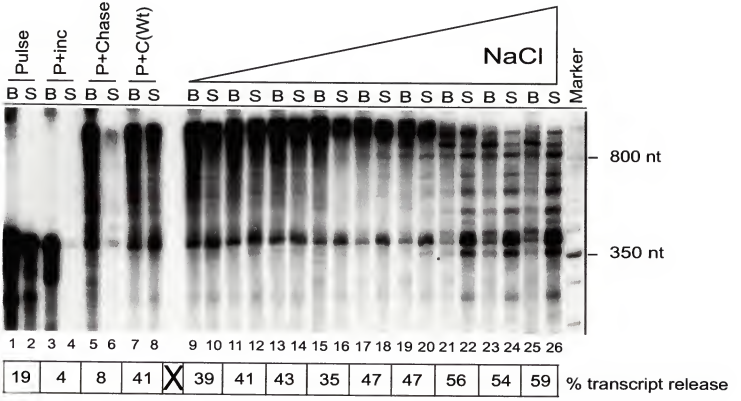

Marker
0.01%
0.025%
0.05%
0.1%
0.2%
0.3%
Sarkosyl
N W N W N W N W N W N W N W
B S B S B S B S B S B S B S B S B S B S B S B S B S B S
Marker
800 nt
350 nt
1 3 5 7 9 11 13 15 17 19 21 23 25 27
2 4 6 8 10 12 14 16 18 20 22 24 26 28
17 40 15 42 10 31 X 100 85 89 88 97 82 78 75
% transcript release

Salt Stability of Transcription Elongation Complexes

To further characterize the elongation complexes we tested the salt sensitivity of elongation under conditions of non-limiting nucleotides. Salt, similar to sarkosyl, also dissociates elongation factors during an elongation reaction and can therefore reveal the presence or absence of other factors important for elongation. Transcription complexes were assembled and initiated during a 30-minute pulse reaction containing Wt extract, bead-bound template, [α-^{32}P] CTP, ATP, UTP, and 3'-OMeGTP, similar to Figure 5. The NpG8G template contains the vaccinia *G8R* intermediate promoter, a 375-nt G-less cassette, and approximately 2.5-kB additional downstream DNA. The presence of 3'-OMeGTP halts the elongation complex at the end of the G-less cassette where the first GTP would be incorporated resulting in the synthesis of an approximately 400-nt transcript. The pulse-labeled ternary complexes were isolated, washed twice in low salt transcription buffer, resuspended in a mixture containing nucleotides, Wt extract, and various concentrations of NaCl, and allowed to continue elongation during a 20-minute chase. After the elongation reaction the beads and supernatant were separated and quantified using a phosphorimager as described in Figure 6.

During the pulse reaction a significant number of transcripts are released into the supernatant (Fig. 7, compare Lanes 1 and 2) due to the long incubation and presence of Wt extract. The bead-bound complexes were removed from the supernatant containing released transcripts and then chased in the absence or presence of additional nucleotides and extract. A 20-minute incubation in the absence of nucleotides and extract (Fig. 7, compare Lanes 3 and 4) or in the presence of only nucleotides (Fig. 7, compare Lanes 5 and 6) results in minimal transcript release. The addition of Wt extract and nucleotides to

Fig. 7. **Salt stability of transcription elongation complexes.** Transcription complexes were assembled and initiated on immobilized NpG8G template containing the vaccinia G8R intermediate promoter as described in Fig. 1 (Pulse, lanes 1 and 2). The isolated, labeled ternary complexes were washed twice in low salt transcription buffer and resuspended in a mixture containing only buffer (P+inc, lanes 3 and 4), or 1 mM ATP, GTP, CTP, and 0.6 mM UTP either alone (P+chase, lanes 5 and 6) or in the presence of 15 mg Wt extract (P+C+(Wt), lanes 7 and 8) and increasing concentrations of NaCl as follows: 25 mM (lanes 9 and 10), 50 mM (lanes 11 and 12), 100 mM (lanes 13 and 14), 200 mM (lanes 15 and 16), 300 mM (lanes 17 and 18), 350 mM (lanes 19 and 20), 400 mM (lanes 21 and 22), 450 mM (lanes 23 and 24), 500 mM (lanes 25 and 26). Elongation continued for 20 min at 30oC. The bead-bound RNA (B) was separated from released RNA (S) using a magnet. The transcripts were analyzed by 6% 8 M urea-PAGE. Bound and released transcripts were quantitated using a PhosphorImager for the whole lane; the quantity of transcripts in the supernatant was divided by the quantity of transcripts on both the beads and in the supernatant and expressed as a percentage in the table below the autoradiogram. X, indicates an empty lane. Sizes, in nt, are shown on the right.


Pulse
P+inc
P+Chase
P+C(Wt)
NaCl
Marker
B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S
800 nt
350 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
19 4 8 41 X 39 41 43 35 47 47 56 54 59
% transcript release

the bead-bound complexes promotes the release of additional transcripts (Fig. 7, compare Lanes 7 and 8). These data indicate that halted ternary complexes isolated using 3'-OMeGTP are capable of continued elongation when complemented with GTP and additional nucleotides. Additionally, the isolated complexes are stable during continued incubation and do not release the nascent RNA until supplemented with extract from Wt-infected cells, indicating that release may be dependent on additional factors not present within the isolated ternary complex. We then tested the salt stability of this reaction by including a titration of NaCl during the chase phase, in addition to the Wt extract and nucleotides (Fig. 7, Lanes 9-26). Two observations are of note: release of the nascent RNA in the presence of Wt extract occurs regardless of the concentration of NaCl and increasing NaCl concentration impairs transcription elongation. The release of nascent RNA does appear to increase slightly with addition of NaCl from 300 mM to 500 mM (Fig. 7, Lanes 17-26). This increase in release is accompanied by a decrease in the elongation potential as evidenced by the appearance of multiple bands representing transcripts shorter than the full-length template (Fig. 7, Lanes 21-26). These data could indicate that the increasing concentration of NaCl is inhibiting the interaction between a positive transcription elongation factor and the ternary complex. In the absence of this factor increased pausing and release of the transcripts may occur.

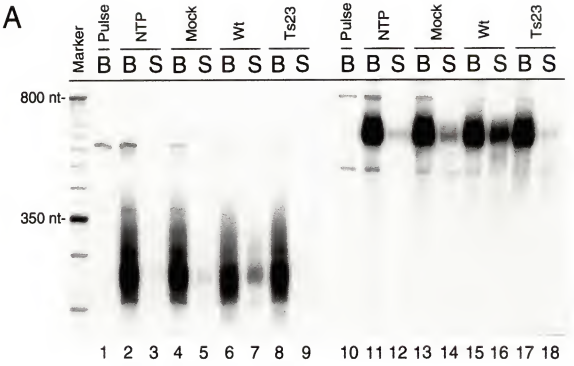
In Vitro Transcription Is Specific for the Viral Promoter

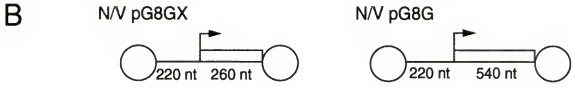
The aforementioned *in vitro* transcription reaction was again modified to decrease the background levels of release seen in the absence of Wt extract. Additionally, we tested the fidelity of the pre-incubation step in the *in vitro* system by proving that the intermediate promoter was accurately recognized. Transcription complexes were

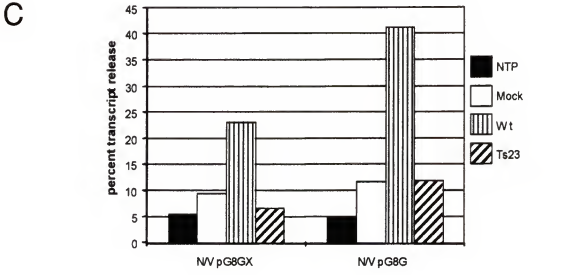
assembled during the pre-incubation reaction containing Wt extract, bead-bound template, and ATP. Transcription was then initiated and the nascent transcript radiolabeled by the addition of [α-32P] CTP, ATP, GTP, and UTP during a short, 30-sec pulse reaction. The ternary complexes were stripped of nonspecific proteins and unincorporated nucleotides during three washes in high salt transcription buffer (1 M KOAc) followed by three washes in low salt transcription buffer (80 mM KOAc). The elongation reaction was performed with the addition of a chase mixture containing NTPs, extract, and proteins. Following the elongation reaction the beads were concentrated using a magnet, the supernatant was removed to a separate tube, and the labeled RNA in each fraction was analyzed on a denaturing polyacrylamide gel.

To prove that the intermediate promoter was accurately recognized, two bead-bound templates were designed such that transcription from the *G8R* promoter to the downstream end of the template would generate either 260-nt or 540-nt of RNA (Fig. 8B). Pulse-labeled elongation complexes were established and analyzed on a denaturing polyacrylamide gel (Fig. 8A, Lanes 1 and 10). The transcripts were approximately 100-nt in length and were cut off on the autoradiograph shown. Elongation was continued on addition of ribonucleotides during the chase phase and the transcripts synthesized from each template were of the appropriate length, either 260-nt or 540-nt (Fig. 8A, Lanes 2 and 11). At the end of the chase phase, the bead-bound template was separated from the supernatant using a magnet. Comparison of Lanes 2 and 3 and Lanes 11 and 12, Fig. 8A, indicate that transcripts synthesized during a nucleotides-only chase reaction are not released into the supernatant but remain associated with the bead-bound template. This newest protocol for generating elongation complexes used extensive washing with 1 M

Fig. 8. **Transcription is promoter-specific.** *A*, autoradiogram of *in vitro* transcript release assay. Transcription complexes were formed from Wt extract on immobilized N/VpG8GX or N/VpG8G DNA that contain the vaccinia *G8R* intermediate promoter. Following a 30-sec pulse reaction (*Pulse*), labeled complexes were washed in transcription buffer, and elongation was continued in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTP*) or with additional 7.5 μg mock extract (*Mock*), Wt extract (*Wt*), or Cts23 extract (*Ts23*) for 20 min. The bead-bound RNA (*B*) was separated from released RNA (*S*) using a magnet. These transcripts were analyzed by 6% 8 M urea-PAGE. Sizes, in nt, are shown on the *left*. *B*, diagram of the DNA templates used for transcription. The DNA template (*line*) contains a biotinylated ATP incorporated at both the 5' and 3' end, which anchors the DNA to a streptavidin-coated magnetic bead (*circles*). The bead is anchored 220 nt from the promoter at the 5' end of the template. The transcription unit consists of the *G8R* intermediate promoter (*arrow*) fused to either 260 or 540 nt of downstream DNA. *C*, graphic representation of the percent transcript release for each reaction in *A*.


A
Marker
I Pulse
NTP
Mock
Wt
Ts23
B B S B S B S B S B S
800 nt-
350 nt-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


B
N/V pG8GX
220 nt 260 nt
N/V pG8G
220 nt 540 nt


C
percent transcript release
45
40
35
30
25
20
15
10
5
0
NTP
Mock
Wt
Ts23
N/V pG8GX
N/V pG8G

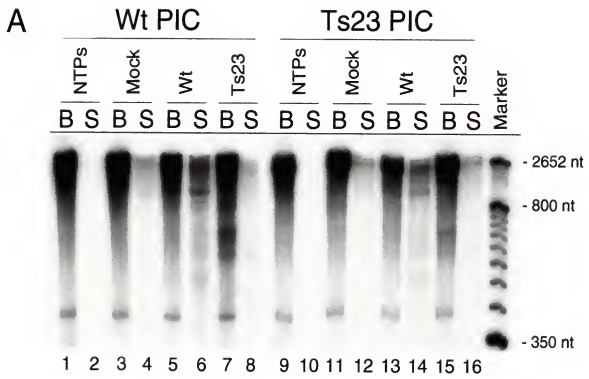
salt and we questioned whether additional proteins could act on the isolated elongation complexes to induce release of the nascent transcript from the bead-bound template as shown in the salt and sarkosyl stability experiments. Chase reactions were therefore performed in the presence of nucleotides plus extract from mock-, Wt-, or the *A18R* mutant Cts23-, infected cells. The addition of extract from Wt-infected cells resulted in the release of transcripts during a 20-minute chase from either template (Fig. 8A,compare Lanes 6 and 7, and Lanes 15 and 16). The percent transcript release was analyzed by phosphorimagery (Fig. 8C). Extract from neither mock-infected nor Cts23-infected cells was capable of generating a significant amount of released transcripts (Fig. 8A, Lanes 4 and 5, 8 and 9, 13 and 14, and 17 and 18, Fig. 8C). In summary, these experiments show that initiation *in vitro* occurs specifically at the viral intermediate promoter. These data also suggest that transcript release in Wt extract is due to the presence of A18 protein, which is absent in Cts23 extract.

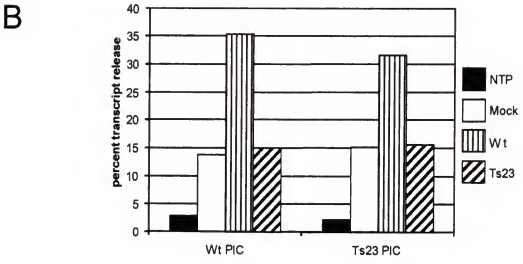
Specific Aim 2: *In Vitro* Analysis of the A18 Phenotype

Release Does Not Require the Presence of A18R during Initiation

In Figures 6 and 8, Wt extract was used to generate the transcription complexes formed during the pre-incubation step. To determine whether factors specific to a Wt extract and present in the washed elongation complex contributed to release, we compared transcription complexes formed using either Wt or Cts23 extract during the pre-incubation and pulse steps (Fig. 9A, Wt or Cts23 PIC). Transcription complexes were formed on linearized bead-bound NpG8G, a template that contains approximately 3 kb of sequence downstream from the *G8R* promoter. After initiation with the addition of nucleotides and a thorough wash in high salt and low salt transcription buffers, these

Fig. 9. **A18 is not required for initiation *in vitro*.** *A*, transcription complexes were formed on immobilized NpG8G DNA and extract from either Wt- (*Wt PIC*) or Cts23 (*Ts23 PIC*)-infected cells. Transcription was performed as described in Fig. 3 and released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed by 6% 8 M urea-PAGE. Sizes in nt are shown at the *right*. *B*, graphic representation of the percent transcript release for each reaction in *A*.


A
Wt PIC
Ts23 PIC
NTPs
Mock
Wt
Ts23
NTPs
Mock
Wt
Ts23
Marker
B S B S B S B S B S B S B S B S B S
- 2652 nt
- 800 nt
- 350 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
percent transcript release
40
35
30
25
20
15
10
5
0
NTP
Mock
Wt
Ts23
Wt PIC
Ts23 PIC

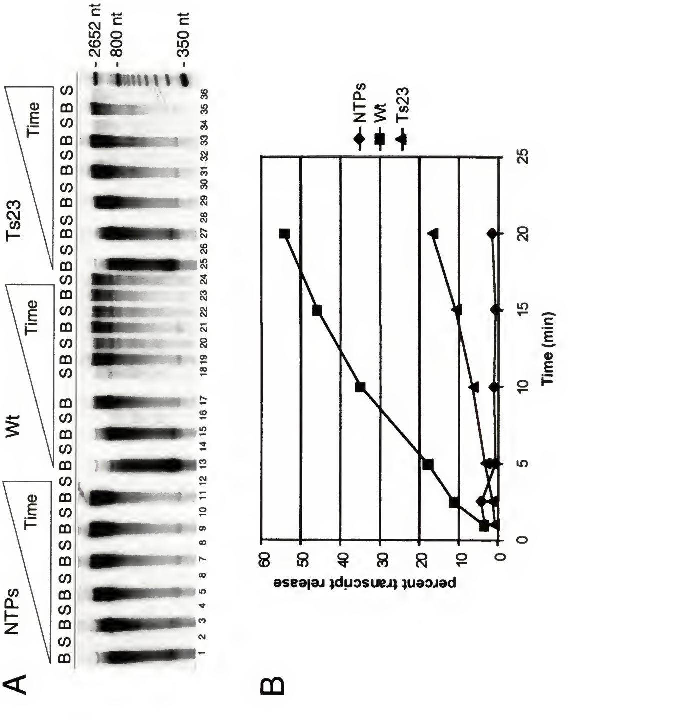
complexes were chased in the presence of unlabeled ribonucleotides, or nucleotides plus mock, Wt, or Cts23 extract (Fig. 9A). Both complexes show similar levels of transcript release in response to the addition of Wt extract (Fig. 9A, compare Lanes 5 and 6, 13 and 14, Fig. 9B). Therefore, Wt or Cts23 extracts are equally competent for transcription complex assembly and initiation. Therefore, Wt extract was used to generate transcription complexes for all release assays.

Transcript Release Is Time and Concentration Dependent

To determine the kinetics of release, we performed a time course of elongation. Pulse-labeled elongation complexes were formed and samples were taken at various time points during elongation. Similar kinetics of elongation were observed with the addition of ribonucleotides alone, or in combination with Wt or Cts23 extract (Fig. 10A). Release is detected with the addition of Wt extract (Fig. 10A, Lanes 13-24) and the level of release increases linearly as a function of time (Fig. 10B). Longer incubation times do not result in more than 60% release. Cts23 extract also resulted in a linear increase in release activity with time that was measurably above the nucleotides-only control but significantly less than Wt (Fig. 10A, Lanes 25-36, Fig. 10B). The lower level of release observed with addition of Cts23 extract could represent non-specific release or result from the lower level of A18 protein in Cts23 extract. In summary, release activity is significantly diminished in a Cts23 extract throughout a time course substantiating our hypothesis that release is specific to the presence of A18 protein.

We then titrated the concentration of extract included in the elongation step to determine the optimal quantity of extract for efficient release. Transcription complexes were formed from Wt extract during the pre-incubation step, initiated with the addition of

Fig. 10. **Time course of elongation in a chase reaction.** *A*, pulse-labeled transcription elongation complexes were formed on NpG8G bead-bound template using extract from Wt-infected cells. Complexes were washed in 1 M transcription buffer, and transcription was continued in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTPs*) or in addition to 30 μg of Wt extract (*Wt*) or Cts23 (*Ts23*) for 1, 2.5, 5, 10, 15, and 20 min. Released transcripts in the supernatant (*S*) and bound transcripts associated with the bead-bound template (*B*) were separated and analyzed by denaturing 6% 8 M urea-PAGE. *B*, graphic representation of the percent transcript release for each reaction in *A*.


A
NTPs
Wt
Ts23
Time
Time
Time
B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
- 2652 nt
- 800 nt
- 350 nt
B
percent transcript release
0
10
20
30
40
50
60
0
5
10
15
20
25
Time (min)
NTPs
Wt
Ts23

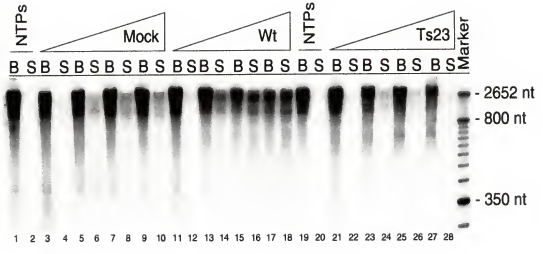
nucleotides, washed in high and low salt transcription buffers, and then assayed for elongation and transcript release using increasing concentrations of mock, Wt, or Cts23 extract and ribonucleotides during a 20-minute chase reaction. Increased transcript release occurred as the quantity of Wt extract was increased (Fig. 11A, Lanes 12-19, Fig.11B), however, no effect on release was observed with increasing quantities of either mock or Cts23 extract (Fig. 11A, Lanes 4-11 and Lanes 23-30, Fig. 11B). These results further support the hypothesis that A18 is important for transcript release.

Transcript Release Is Complemented by Crude Fractions from Wt Extract

In an attempt to correlate the release activity achieved by the addition of Wt extract with the presence of A18 protein, a crude fractionation protocol was employed. Extracts were prepared from either Wt- or Cts23-infected cells and fractionated on phosphocellulose and Q-Sepharose columns separately. Columns were eluted step-wise with 0.25 M, 0.5 M, and 1 M NaCl. Each Wt extract fraction was analyzed by SDS-PAGE (data not shown) and by western blot analysis using an anti-A18 monoclonal antibody (Fig. 12B and C). As demonstrated by western blot, A18 protein fractionated into the 0.5 M phosphocellulose fraction and the 0.25 M Q-Sepharose fraction (Fig. 12B, 0.5 M and Fig. 12C, 0.25 M). Each fraction was assayed for its ability to induce transcript release using the protocol described in Figure 11 (Fig. 12A). As controls, elongation reactions containing ribonucleotides alone, or ribonucleotides plus mock, Wt, or Cts23 extract were performed (Fig. 12A, Lanes 1-8, 13 and 14). As previously shown, only the addition of Wt extract is capable of inducing transcript release (Fig. 12A, Lanes 5 and 6 and Lanes 13 and 14, Fig. 9D). Two of the column fractions were capable of inducing release, the 0.5 M phosphocellulose fraction and the 0.25 M Q-Sepharose

Fig. 11. **Add-back extract titration.** *A*, elongation complexes were generated as detailed in Fig. 10, washed in 1 M transcription buffer, and transcription was continued for 20 min in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTPs*). Other reactions were supplemented with increasing concentrations of mock extract (*Mock*), Wt extract (*Wt*), or Cts23 extract (*Ts23*), as follows: 0.5 μg (*lanes 4* and *5*, *12* and *13*, and *23* and *24*), 3 μg (*lanes 6* and *7*, *14* and *15*, and *25* and *26*), 15 μg (*lanes 8* and *9*, *16* and *17*, and *27* and *28*), 30 μg (*lanes 10* and *11*, *18* and *19*, and *29* and *30*). *B*, bound; *S*, supernatant. *B*, percent transcript release plotted against the quantity of mock, Wt, or Cts23 extract.

A


NTPs
Mock
Wt
NTPs
Ts23
Marker
B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S B S
- 2652 nt
- 800 nt
- 350 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

B

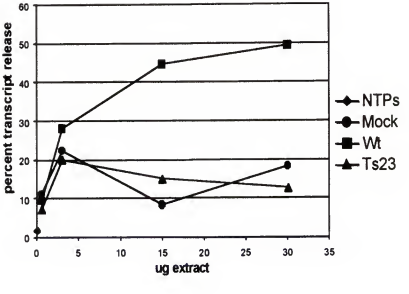
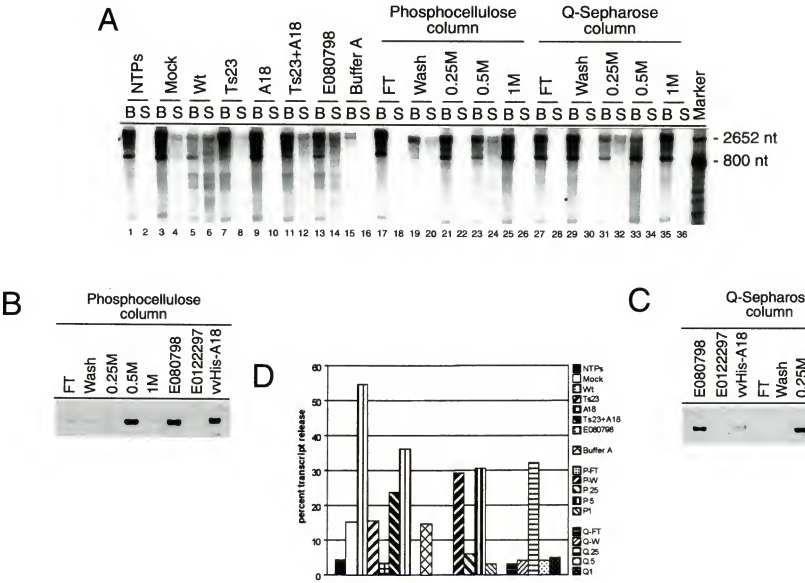

percent transcript release
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
ug extract
NTPs
Mock
Wt
Ts23

Fig. 12. **Wt extract fractionation.** *A*, pulse-labeled elongation complexes were generated as detailed in Fig. 10. Transcript release was assayed with the addition of 0.6 mM ATP, GTP, UTP, and 1.2 mM CTP (*NTPs*) or NTPs and 30 μg of mock (*Mock*), Wt (*Wt* and *E080798*), or Cts23 (*Ts23*) extract, 1.32 μg of vaccinia virus (*vv*) His-A18 protein (*A18*), or 5 μg of each fraction from the phosphocellulose and Q-Sepharose columns during a 20-min chase reaction. E080798 was the extract fractionated on the phosphocellulose and Q-Sepharose columns. *B*, bound; *S*, supernatant. *B* and *C*, Western blot analysis. Monoclonal α-A18 antibody was used to probe a 10% SDS-PAGE containing 3.125 μg of each sample from the phosphocellulose and Q-Sepharose columns, 7.5 μg either Wt extract (*E080798*) or Cts23 extract (*E122297*), and 0.3 μg of purified vvHis-A18 protein. *D*, graphic representation of the percent transcript release for each sample in *A*.


A
Phosphocellulose column
Q-Sepharose column
NTPs
Mock
Wt
Ts23
A18
Ts23+A18
E080798
Buffer A
FT
Wash
0.25M
0.5M
1M
FT
Wash
0.25M
0.5M
1M
Marker
B S
2652 nt
800 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
B
Phosphocellulose column
FT
Wash
0.25M
0.5M
1M
E080798
E0122297
vvHis-A18
C
Q-Sepharose column
E080798
E0122297
vvHis-A18
FT
Wash
0.25M
0.5M
1M
D
percent transcript release
0
10
20
30
40
50
60
NTPs
Mock
Wt
Ts23
A18
Ts23+A18
E080798
Buffer A
P-FT
P-W
P.25
P.5
P1
Q-FT
Q-W
Q.25
Q.5
Q1

fraction (Fig. 12A, Lanes 23 and 24 and Lanes 31 and 32). These same fractions contain A18 protein as judged by western blot analysis (Fig. 12B, 0.5M and Fig. 12C, 0.25M). The phosphocellulose wash fraction, Fig. 12A, Lanes 19 and 20, also showed release in this experiment. This result was not reproducible in subsequent release experiments done with the same material. For comparison, Cts23 extract was also fractionated by the same protocol (data not shown). A18 protein was not detected by western blot in extract from Cts23-infected cells (Fig. 12B, E122297), nor any Cts23 extract fractions from the phosphocellulose or Q-Sepharose columns (data not shown). In addition, no significant release was detected with the addition of fractions from Cts23 extract (data not shown). The fractionation protocol described here provides circumstantial evidence for the role of A18 protein in transcript release. However, these are crude fractions that contain many more proteins than just A18. Conclusive evidence for the role of A18 must be obtained with a purified fraction or purified protein.

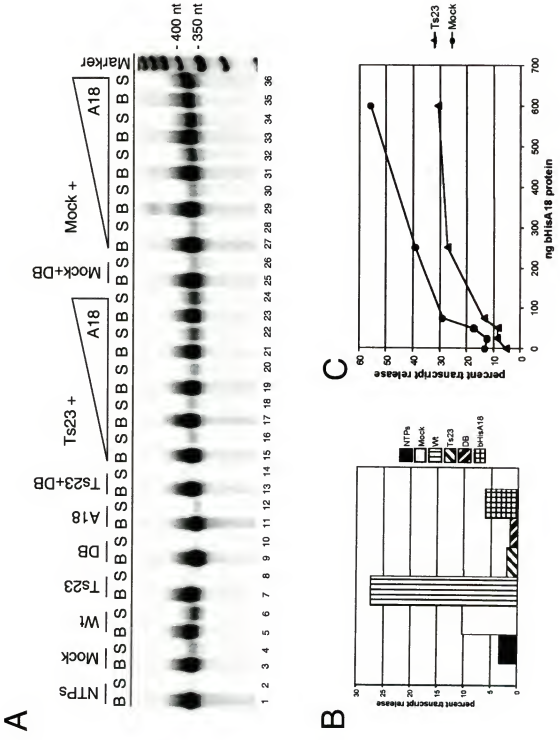
Release Occurs From a Stalled Elongation Complex and Can Be Complemented by His-A18 and a Cellular Factor

In all of the experiments described above release occurs predominately at the downstream end of the template where the template is joined to a paramagnetic bead. In order to eliminate the possibility that the observed release is an artifact due to the presence of the bead, we conducted experiments designed to promote release in the middle of a DNA template. We refer to this protocol as a "mid-template" assay. This assay is designed to reflect the situation *in vivo* where a transcription complex will terminate despite the presence of additional template downstream. We accomplished this by arresting transcription at the end of a 375-nt G-less cassette downstream from the intermediate *G8R* promoter present within the 3-kB NpG8G template. Transcription

complexes were assembled on NpG8G during the pre-incubation reaction, pulse-labeled, washed in high salt transcription buffer, and elongated either in the absence of GTP (with all other nucleotides present) (data not shown) or in the presence of 3'-OMeGTP and all other ribonucleotides (Fig. 13A) with additional proteins provided as indicated. The addition of 3'-OMeGTP arrests the elongation complex at the end of the G-less cassette where the first GTP is incorporated (Fig. 13A, Lane 1) resulting in the synthesis of an approximately 400-nt transcript. Addition of Wt extract during the chase reaction resulted in release of the transcript at the end of the G-less cassette (Fig. 13A, Lanes 5 and 6). Release did not occur with mock or Cts23 extract (Fig. 13A, Lanes 3 and 4, Lanes 7 and 8). Similar results were obtained when the complex was elongated in the absence of GTP (data not shown). In other experiments not shown, we attempted to induce release by first elongating to the end of the G-less cassette in the absence of added extract and then adding extract to the arrested complex for an additional incubation. We also tried to induce mid-template release by slowing elongation using reduced concentrations of UTP. In neither protocol did we observe significant mid-template release. These results show definitively that release can be induced in the middle of the template but strongly suggest that release can only be accomplished on a complex that is stalled. Furthermore, the results indicate that in order to observe release, release factors need to be present during elongation, before the polymerase stalls.

In order to determine definitively whether A18 is required for transcript release, we attempted to complement the defect in release activity observed in Cts23 extracts with the addition of purified His-A18 protein. Pulse-labeled elongation complexes were formed and assayed for transcript release during an elongation step using purified His-

Fig. 13. **Release occurs from a stalled elongation complex and can be complemented by His-A18 and a cellular factor.** *A*, pulse-labeled transcription elongation complexes were formed on NpG8G bead-bound template using extract from Wt-infected cells. Complexes were washed in 1 M transcription buffer, and transcription elongation was continued to the end of the G-less cassette using 0.6 mM ATP, UTP, 1.2 mM CTP, and 0.01 mM 3'-OMeGTP alone (*NTPs*), or in addition to 30 μg of mock-infected extract (*Mock*), Wt extract (*Wt*), or Cts23 extract (*Ts23*). Transcripts synthesized in the presence of 3'-OMeGTP are approximately 400 nt in length. Purified recombinant His-A18 protein was used at 300 ng either alone (*A18*) or in combination with Cts23 or mock extract. *DB*, A18 protein storage buffer; *B*, bound; *S*, supernatant. *B* and *C*, graphic representation of the percent transcript release for each sample in *A*


A
NTPs
Mock
Wt
Ts23
DB
A18
Ts23+DB
Ts23 +
A18
Mock+DB
Mock +
A18
Marker
- 400 nt
- 350 nt
B
percent transcript release
NTPs
Mock
Wt
Ts23
DB
bHisA18
C
percent transcript release
ng bHisA18 protein
Ts23
Mock

A18 protein. His-A18 was expressed in *E. coli* and purified over nickel and phosphocellulose columns as described in Chapter 2. The addition of ribonucleotides, Cts23 extract, or purified His-A18 protein alone to the chase was not sufficient for transcript release (Fig. 13A, Lanes 11 and 12, Fig. 13B). Addition of increasing amounts of His-A18 protein to the Cts23 extract resulted in increasing release equivalent to the levels of His-A18 protein (Fig. 13A, Lanes 15-24, Fig. 13C). As a control, a similar titration of purified His-J3 protein (J3 is the vaccinia 2'-O-methyltransferase and poly(A) polymerase processivity factor) expressed in *E. coli* was tested in combination with Cts23 extract (data not shown). The transcription complexes did not release the nascent RNA in the presence of His-J3 protein. These results demonstrate that the release defect observed in Cts23 extract can be complemented by purified A18 protein.

The results described above show that purified A18 protein is necessary but not sufficient for transcript release. To determine whether the additional factors required for release are viral or cellular in nature, extract from mock-infected cells was tested in the release assay. Mock extract alone does not produce a significant level of released transcripts (Fig. 13A, Lanes 3 and 4). A titration of His-A18 in combination with mock extract induced more efficient release than His-A18 plus Cts23 extract (Fig. 13A, compare Lanes 27-36 and Lanes 15-24, Fig. 13C). The simplest explanation for these observations is that a cellular factor(s) is needed in addition to A18 for transcript release.

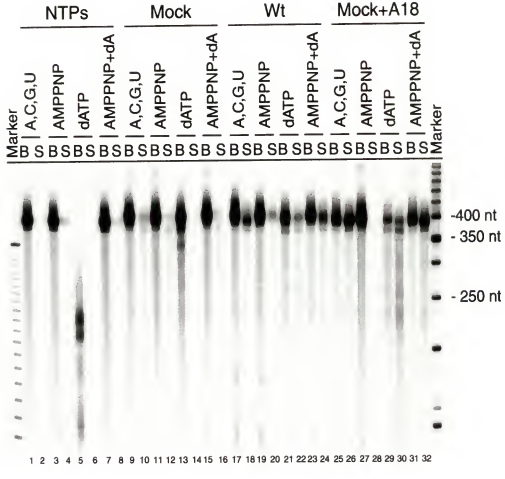
Release Requires ATP Hydrolysis

It was shown previously that A18 possesses a DNA-dependent ATPase activity and that the enzyme can readily use dATP as a substrate rather than ATP (9). We therefore hypothesize that any stage of transcription that requires A18 would also be

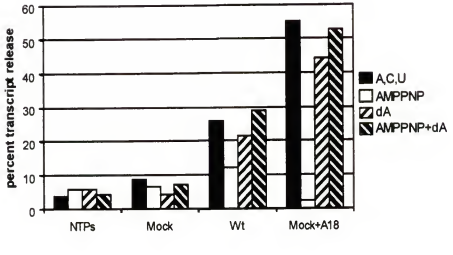
ATP-dependent. Assessing the role of ATP hydrolysis in transcription is complicated by the requirement for ATP as a substrate for the polymerase during elongation. Therefore, we examined the ATP-dependence of the release activity by replacing the ATP in the elongation step of the mid-template assay with the non-hydrolyzable ATP analog, AMPPNP. AMPPNP can be used as a substrate for the vaccinia RNA polymerase and substitution results in efficient synthesis of long transcripts (Fig. 14A, compare Lanes 1 and 3). Substitution of ATP with dATP, a hydrolyzable ATP analog that cannot be efficiently used for synthesis, yielded transcripts that are much shorter in length (Fig. 14A, compare Lanes 1 and 5). Transcription elongation in the presence of dATP can be rescued with the provision of AMPPNP (Fig. 14A, Lane 7). The combination of dATP and AMPPNP satisfies the energy requirement and provides a nucleotide capable of being incorporated into the nascent RNA chain. We then assayed the effect of AMPPNP substitution on release in combination with mock extract (Fig. 14A, Mock), Wt extract (Fig. 14A, Wt), or mock extract plus His-A18 protein (Fig. 14A, Mock+A18). As controls, the level of release in response to a given extract was assayed using ATP or dATP alone or the combination of AMPPNP and dATP, and quantified as previously described (Fig. 14A, Lanes 9 and 10, 15 and 16, 17 and 18, 23 and 24, 25 and 26, and 31 and 32, Fig. 14B). Since the extract added during the elongation step contains some endogenous ATP, substitution of ATP with dATP in these controls did not restrict elongation as much as elongation in the presence of nucleotides alone. Substitution of ATP with AMPPNP did not have an effect on the low level of release detected in the presence of mock extract (Fig. 14A, compare Lanes 9 and 10 and Lanes 11 and 12, Fig. 14B). On the other hand, replacing ATP with AMPPNP severely inhibits transcript

Fig. 14. **Transcript release requires ATP hydrolysis.** *A*, ternary complexes were formed and elongated as described in Fig. 9. The standard elongation reaction included 0.6 mM ATP, UTP, 0.01 mM 3'-OMeGTP, and 1.2 mM CTP (*A, C, G, U*). In other reactions, adenosine analogs AMPPNP (*AMPPNP*) and dATP (*dA* or *dATP*) replaced ATP as indicated, each at 0.6 mM concentration. Released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed as described previously. *B*, graphic representation of the percent transcript release for each sample in *A*.

A


NTPs
Mock
Wt
Mock+A18
Marker
A,C,G,U
AMPPNP
dATP
AMPPNP+dA
B S
-400 nt
- 350 nt
- 250 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

B


percent transcript release
60
50
40
30
20
10
0
NTPs
Mock
Wt
Mock+A18
A,C,U
AMPPNP
dA
AMPPNP+dA

release when assayed with Wt extract (Fig. 14A, compare Lanes 17 and 18 and Lanes 19 and 20, Fig. 14B) or mock extract plus His-A18 protein (Fig. 14A, compare Lanes 25 and 26 and Lanes 27 and 28, Fig. 14B). Therefore, we conclude that A18 catalyzed transcript release is an ATP-dependent event.

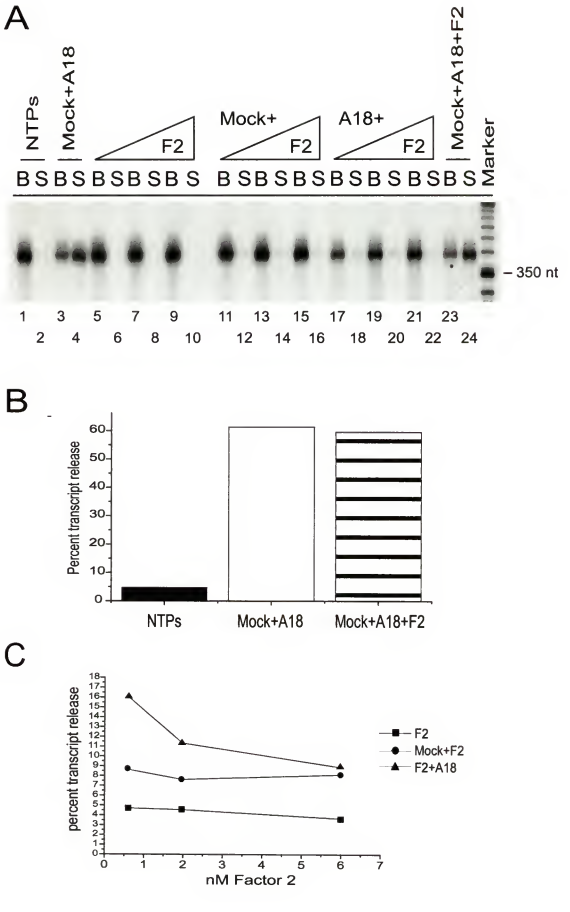
Specific Aim 3: Characterization of the Cellular Factor

Cellular Factor is not Human Factor 2

Human Factor 2 is well characterized and currently the only identified eukaryotic RNAPII transcript release factor (84,165). This factor was identified based on its involvement during the pre-initiation stage of transcription and its activity in release of transcripts from RNAPII early elongation complexes. Factor 2 is a strong DNA-dependent ATPase and possesses a helicase motif although helicase activity has not been detected (164). Recent data indicates that Factor 2 is also able to disrupt RNAPII, as well as RNAPI, ternary complexes stalled at a thymine cyclobutane dimer (54). The discovery of a transcription elongation factor with activity on different classes of RNAP is not unprecedented. In addition to Factor 2, TFIIS was shown to cause transcript cleavage during both RNAPI and RNAPII transcription elongation (137). Therefore, we sought to determine whether Factor 2 was involved in transcript release from vaccinia promoters and specifically whether Factor 2 would substitute for CF activity.

Using the mid-template assay, pulse-labeled elongation complexes were formed and assayed for transcript release during the elongation step using His-A18 protein and mock extract, Factor 2 alone, Factor 2 and mock extract, or His-A18 and Factor 2 as indicated (Fig. 15). The addition of mock extract and His-A18 provides the positive control demonstrating high levels of transcript release (Fig. 15A, Lanes 3 and 4, Fig.

Fig. 15. **Factor 2 is not the cellular factor.** *A*, transcription complexes were assembled and initiated as described in Fig. 13. Elongation was continued to the end of the G-less cassette using 0.6 mM ATP, UTP, 1.2 mM CTP, and 0.01 mM 3'-OMeGTP alone (*NTPs*), or in addition to 3 μg Mock extract (*Mock*), 600 ng His-A18 (*A18*), and/or Factor 2 (*F2*) at 0.6 mM, 2 nM, or 6nM as indicated for 20 min. Released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed as described previously. *B* and *C*, graphic representation of the percent transcript release for each sample in *A*.


A
NTPs
Mock+A18
F2
Mock+
F2
A18+
F2
Mock+A18+F2
Marker
B S B S B S B S B S B S B S B S B S B S B S B S B S B S
– 350 nt
1 3 5 7 9 11 13 15 17 19 21 23
2 4 6 8 10 12 14 16 18 20 22 24
B
Percent transcript release
60
50
40
30
20
10
0
NTPs
Mock+A18
Mock+A18+F2
C
percent transcript release
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
nM Factor 2
F2
Mock+F2
F2+A18

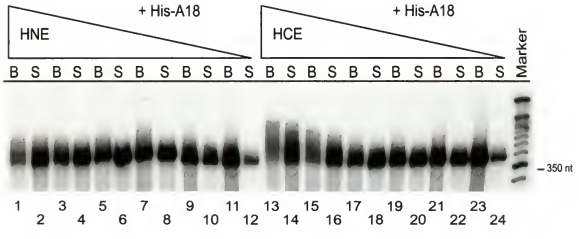
15B). Transcript release is neither increased nor decreased with the addition of Factor 2 (Fig. 15A, Lanes 23 and 24, Fig. 15B). A titration of Factor 2 alone, or in the presence of either mock extract or His-A18 protein demonstrates no release of nascent RNA into the supernatant (Fig. 15A, Lanes 5-22, Fig. 15C). Therefore, we conclude that Factor 2 is not involved in release of nascent RNA from vaccinia intermediate promoters and cannot substitute for the unidentified cellular factor.

Cellular Factor Is Present in HeLa Cell Nuclear and Cytoplasmic Fractions

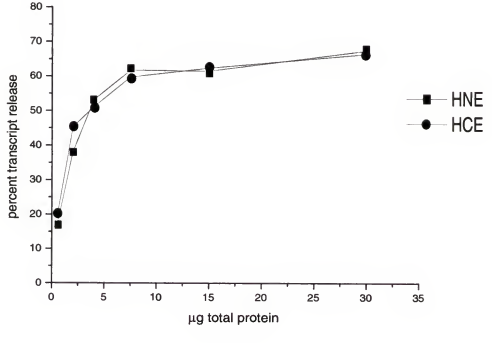
In Figure 13, we demonstrate that the activity required for A18-dependent transcript release is provided by extract from mock-infected A549 cells. As a prerequisite to purification of the cellular factor we looked for the activity in nuclear and cytoplasmic extracts of uninfected HeLa spinner cells (prepared as described in Chapter 2). The localization of activity to either the nuclear or cytoplasmic fraction would aid in purification. Using the mid-template assay, transcript release activity was assayed using a titration of nuclear (Fig. 16A, Lanes 1-12) or cytoplasmic (Fig. 16B, Lanes 13-24) extract in combination with His-A18 protein during the elongation step. Both nuclear and cytoplasmic extracts are capable of inducing transcript release and show similar specific activities (Fig. 16B). However, preparation of the two extracts yields a difference in total protein concentration. Approximately 1.7 mg of total protein is present in the nuclear extract as compared to 15 mg of total protein in the cytoplasmic extract. Therefore, we chose to purify the cellular activity from HeLa cell cytoplasmic extract (HCE).

Fig. 16. **Cellular factor is present in HeLa cell nuclear and cytoplasmic fractions.** A, pulse-labeled elongation complexes were generated as described in Fig. 13. Elongation was performed using the standard nucleotide concentrations, 600 ng His-A18, and extract from either HeLa cell nuclei (*HNE*) or HeLa cell cytoplasm (*HCE*) at 30 μg (*lanes* 1 and 2, 13 and 14), 15 μg (*lanes* 3 and 4, 15 and 16), 7.5 μg (*lanes* 5 and 6, 17 and 18), 4 μg (*lanes* 7 and 8, 19 and 20), 2 μg (*lanes* 9 and 10, 21 and 22), 0.5 μg (*lanes* 11 and 12, 23 and 24) for 20 min. Released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed as described previously. *B*, graphic representation of the percent transcript release for each sample in *A*.

A


+ His-A18
HNE
+ His-A18
HCE
Marker
B S B S B S B S B S B S B S B S B S B S B S B S
– 350 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

B


percent transcript release
µg total protein
HNE
HCE
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35

Cellular Factor Activity Is Inactivated by Heat

To demonstrate that the cellular activity necessary for A18-dependent release is a protein, a heat inactivation experiment was performed. Samples of HCE were incubated at 37°C, 50°C, or 65°C for 10, 30, or 60 minutes. The heat-incubated extract was placed on ice and assayed for *in vitro* transcript release activity in the mid-template assay in combination with His-A18 protein. The release activity of HCE was stable at 37°C for up to 60 minutes (Fig. 17A, compare Lanes 1 and 2 to Lanes 3-8, Fig. 17B). In contrast, incubation of HCE at either 50°C or 65°C for only 10 minutes resulted in a sharp decrease in release activity (Fig. 17A, Lanes 9 and 10 and Lanes 15 and 16, Fig. 17B). Further experimentation indicates that tRNA does not substitute for HCE in the release assay (data not shown). Our current data suggest that the activity provided by a HeLa cytoplasmic extract is a protein.

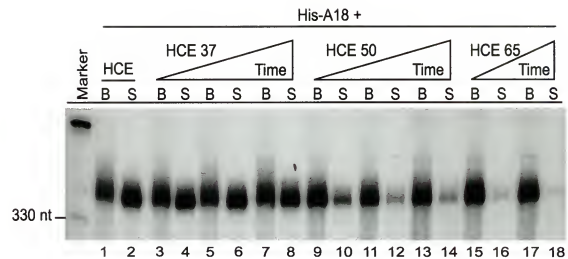
Purification of the Cellular Factor

Attempts at purification have involved the use of conventional chromatography techniques including ion-exchange, hydroxyapatite, hydrophobic interaction, and heparin agarose. Information regarding the nature of the cellular activity has been obtained although attempts at purification are still in progress.

Cytoplasmic extract was prepared from uninfected HeLa cells and fractionated on a High-Q (HQ) anion exchange column. The column was eluted using a gradient from 0 to 0.5 M NaCl at pH 6, followed by a 2 M NaCl wash to elute remaining bound protein. The peak protein fractions (determined by A_{280}) were analyzed by Silver Stain (data not shown) and assayed for transcript release activity (in reactions that contained His-A18 protein). As shown in Fig. 18B, the transcript release activity elutes in a broad peak

Fig. 17. **Cellular factor is inactivated by heat.** *A*, HCE was either untreated or incubated at 37°C, 50°C, or 65°C for 10, 30, or 60 min (*Time*) and placed on ice. Transcription elongation complexes were assembled and initiated as described in Fig 13. Elongation was performed with the addition of the standard nucleotide concentrations, 150 ng His-A18, and 3.2 μg HCE either untreated (*lanes* 1 and 2) or previously incubated at 37°C (*HCE 37, lanes* 3-8), 50°C (*HCE 50, lanes* 9-14), or 65°C (*HCE 65, lanes* 15-18) for 20 min. Released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed as described previously. *B*, graphic representation of the percent transcript release for each sample in *A*.

A


His-A18 +
Marker
HCE
HCE 37
Time
HCE 50
Time
HCE 65
Time
B S B S B S B S B S B S B S B S B S
330 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

B

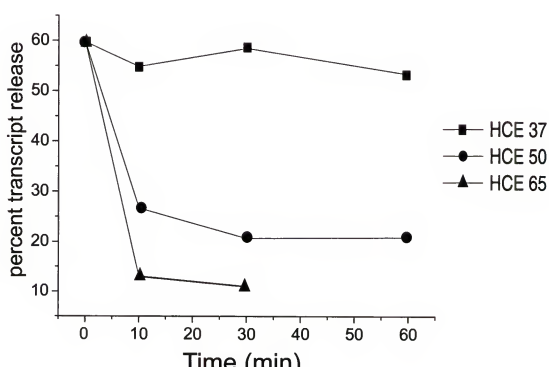
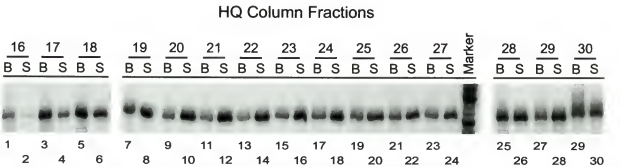
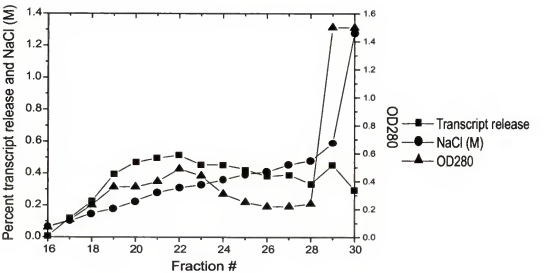

percent transcript release
60
50
40
30
20
10
0 10 20 30 40 50 60
Time (min)
HCE 37
HCE 50
HCE 65

Fig. 18. **Anion exchange chromatography.** *A*, pulse-labeled elongation complexes were generated as detailed in Fig. 9. Transcript release was assayed with the addition of 0.6 mM ATP, GTP, UTP, and 1.2 mM CTP, 150 ng His-A18 protein, and 3 µl of each fraction 17-30 from a HQ column during a 20-min chase reaction. *B*, bound; *S*, released. *B*, graphic representation of the column fractionation data. The column was eluted using a gradient from 0-0.5 M NaCl. The protein concentration of each fraction was measured using an A_{280}. Cellular factor activity was measured in the *in vitro* transcript release assay and expressed as transcript release, determined by dividing the amount of RNA in the supernatant by the total amount of RNA in the bead and supernatant lanes.

A


HQ Column Fractions
16 17 18 19 20 21 22 23 24 25 26 27 Marker 28 29 30
B S B S B S B S B S B S B S B S B S B S B S B S B S
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

B


Percent transcript release and NaCl (M)
OD280
Fraction #
Transcript release
NaCl (M)
OD280

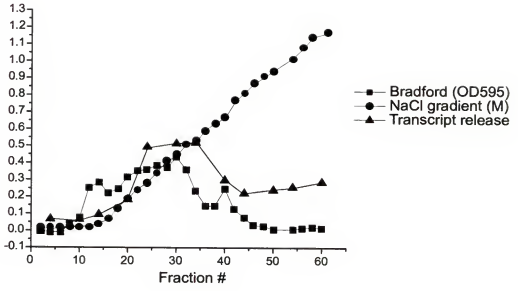
distributed in fractions 19 through 30. Several protein peaks are observed but to do not correlate with a peak of release activity. Several concentrations of each fraction were assayed but did not result in a change in the shape of the activity profile although the absolute values of release did change (data not shown). The column does appear to be separating protein as evidenced by distinct separation of proteins by Silver Stain analysis (data not shown). Numerous purification attempts using anion exchange columns have resulted in similar results (for example, Fig. 19 and data not shown).

Cytoplasmic extract from uninfected HeLa cells was fractionated on a Q-Sepharose column (equivalent to HQ). Individual fractions were pooled, dialyzed and assayed for transcript release activity (in reactions containing His-A18) (Fig. 19A). The pooled fractions E, F, and G (representing the 14-0.5 mL fractions 21 to 35) contained high levels of release activity (Fig. 19A). The individual Q-Sepharose fractions 21 to 35 were pooled and rechromatographed over a hydroxyapatite column. The individual fractions eluted from the hydroxyapatite column were pooled and assayed for transcript release activity. The release activity clearly binds to hydroxyapatite as no activity was detected in the fractions collected prior to implementation of the gradient. However, similar to the results obtained using both HQ and Q-Sepharose columns, the activity elutes in a broad peak, in this case spanning from approximately fractions 22 to 60 (Fig. 19B).

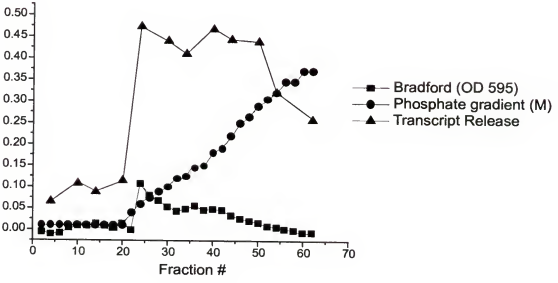
Another attempt at purification employed an initial purification over DEAE-cellulose, a weak anion exchanger, followed by fractionation over phosphocellulose. Previous data indicated that CF bound to the strong anion exchangers HQ and Q-Sepharose so a simple purification on DEAE was used to remove potential nucleic acids

Fig. 19. **Hydroxyapatite Fractionation.** *A*, graphic representation of the Q-Sepharose fractionation data. HCE was bound to Q-Sepharose and fractions were collected during an elution from 0-1 M NaCl. The protein concentration of each fraction was measured using a Bradford protein assay. Groups of five fractions were pooled and assayed for cellular factor activity using the *in vitro* transcript release assay. Activity is expressed as transcript release as described in Fig. 18. *B*, graphic representation of the hydroxyapatite fractionation data. Pooled Q-Sepharose fractions E, F, and G contained the highest level of release activity. The individual fractions represented by these pools (fractions 21-35) were bound to hydroxyapatite and eluted using a 0-.4 M phosphate gradient. The protein concentration of each fraction was measured in a Bradford protein assay. Groups of five fractions were pooled and assayed for activity in the *in vitro* transcript release assay. Activity is expressed as transcript release.

A


1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
10
20
30
40
50
60
Fraction #
Bradford (OD595)
NaCl gradient (M)
Transcript release

B


0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
Fraction #
Bradford (OD 595)
Phosphate gradient (M)
Transcript Release

that may compete with CF for binding to phosphocellulose. Briefly, uninfected HeLa cytoplasmic extract was bound to a DEAE column and eluted in one step at 0.5 M NaCl. Transcript release activity was detected in the single fraction from the DEAE column (data not shown). This fraction was dialyzed and applied to a phosphocellulose column. The column was developed with a gradient and 0.5-ml fractions were collected. The column fractions were pooled and assayed in combination with His-A18 during the elongation step of the mid-template release assay. The release activity was not retained on the phosphocellulose column but rather eluted in the wash phase of the column (Fig. 20).

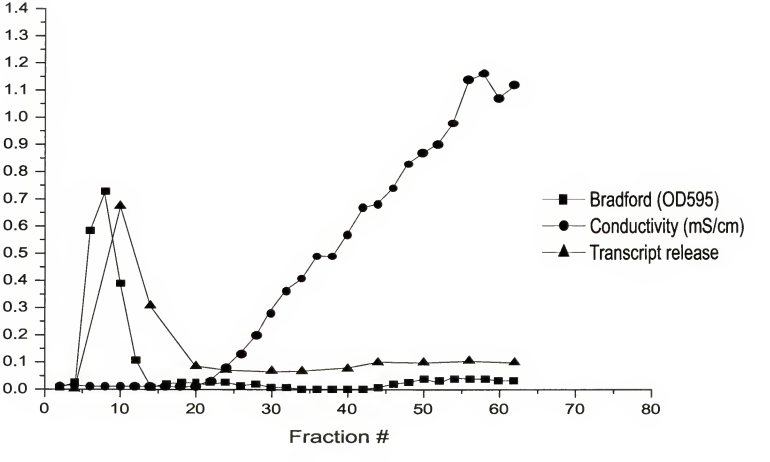
These data indicate that the cellular factor binds to anion exchange columns but does not bind to cation exchange columns. We also attempted to bind the cellular factor to a hydrophobic-interaction column and heparin agarose (data not shown). To date the results using these columns are inconclusive.

Specific Aim 4: Characterize A18/CF-Dependent Release from All Vaccinia Promoters

A18-Dependent Transcript Release Occurs from All Vaccinia Promoters

The previous experiments were all carried out using a template that contained a vaccinia intermediate promoter. However, further characterization of the mechanism by which A18 functions in transcription termination requires analysis of the specificity of the release activity for the different stages of transcription. A18-dependent transcript release assays were performed using templates that contain a promoter from each of the three stages of transcription. Each template is precisely analogous to the intermediate promoter-containing template described previously and contains a 375-nt G-less cassette

Fig. 20. **Phosphocellulose fractionation.** HCE was fractionated over DEAE-cellulose and eluted with 0.5 M NaCl. The eluate was then bound to phosphocellulose and eluted in a gradient from 0-1 M NaCl as measured by conductivity. Fractions were collected and assayed for the flow-through and washes, as well as the gradient fractions. The protein concentration of each fraction was measured using a Bradford protein assay. Individual fractions were pooled as described in Fig. 19 and assayed for activity using the *in vitro* transcript release assay. Activity is expressed as transcript release.


1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
Fraction #
Bradford (OD595)
Conductivity (mS/cm)
Transcript release

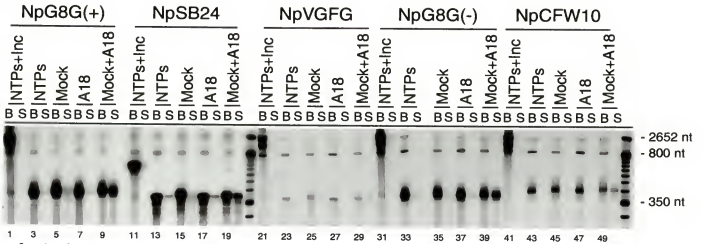
downstream from an early, intermediate, or late gene promoter. In order to assay transcription from early and late promoter driven initiation complexes we used extract from Wt-infected cells that were not treated with hydroxyurea as a source of activity for forming pulse-labeled elongation complexes. Intermediate promoter driven complexes can be formed using extract from either hydroxyurea-treated or non-hydroxyurea-treated Wt-infected cells. The mid-template assay was performed using complexes initiated from each promoter and elongated in the presence of ATP, CTP, UTP, and 3'-OMeGTP, as well as additional proteins. The ability of each complex to release the nascent RNA was determined using mock extract plus His-A18 protein. In the case of each promoter, release occurred only in the presence of both mock extract and His-A18 protein (Fig. 21A, Lanes 9 and 10, 19 and 20, 29 and 30, 39 and 40, 49 and 50, Fig. 21B). Although the absolute level of transcription in non-hydroxyurea extract is less than the hydroxyurea extract, the amounts of released RNA observed from the intermediate promoter template are equivalent (Fig. 21A and Fig. 21B, NpG8G(+) and NpG8G(-)). Additionally, the absolute level of transcription using the various promoters is different but release does occur and is specific for the presence of mock extract plus His-A18 protein. These results indicate that the activity provided by mock extract and His-A18 protein acts on complexes initiated from all three promoters, early, intermediate, and late, implying that A18 could serve as a release factor at each stage in vivo.

CF Enhances Release of Terminated Transcripts Initiated from an Early Promoter

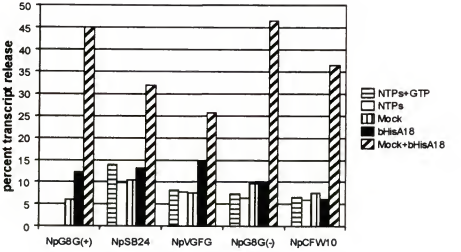
Results indicating a role for A18 and CF in early transcript release are enticing due to the fact that A18 is present in virions, yet no activity for A18 was previously attributed to early gene transcription (146). There is also no previous report of the

Fig. 21. **A18-dependent transcript release occurs from all vaccinia promoters.** *A*, transcription elongation complexes were formed as described in Fig. 13 using templates containing an early promoter (*NpSB24* and *NpVGFG*), an intermediate promoter (*NpG8G*), or a late promoter (*NpCFW10*). The (+) indicates that the transcription complexes were generated using extract from hydroxyurea-treated Wt-infected cells. The (-) indicates that the transcription complexes were generated using extract from non-hydroxyurea-treated Wt-infected cells. Release was measured following an elongation reaction in the presence of the standard nucleotide concentrations (*NTPs*), or nucleotides plus Mock extract (*Mock*) and/or His-A18 protein (*A18*). *B*, bound; *S*, released. *B*, released transcripts are expressed as percent transcript release.

A


NpG8G(+)
NpSB24
NpVGFG
NpG8G(-)
NpCFW10
NTPs+Inc
NTPs
Mock
A18
Mock+A18
B S
- 2652 nt
- 800 nt
- 350 nt

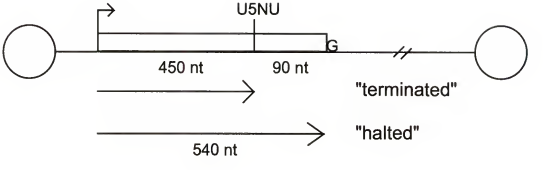
B


percent transcript release
0
5
10
15
20
25
30
35
40
45
50
NpG8G(+)
NpSB24
NpVGFG
NpG8G(-)
NpCFW10
NTPs+GTP
NTPs
Mock
bHisA18
Mock+bHisA18

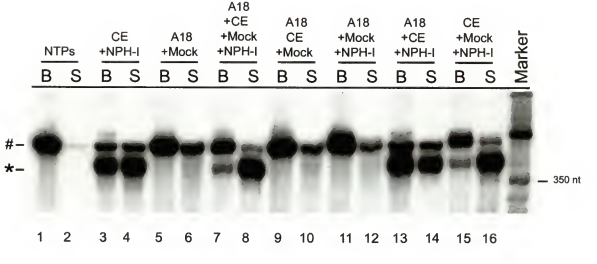
necessity of a host cell factor for early transcription. We therefore sought to further define the role of A18 and CF in early transcript release by using the vaccinia proteins CE and NPH-I required for termination in response to the early termination signal (28,42,94). Using a template that contains the vaccinia virus synthetic early promoter driving a G-less cassette encoding the early termination signal (Fig. 22A), we can differentiate between an effect on specific early transcript termination and release of nascent RNA at the end of a G-less cassette. It should be noted that "termination" in the early system is defined by transcription complexes that do not transcribe past the termination signal, regardless of whether the transcript is bead-bound or released into the supernatant. For the purposes of this assay, transcripts that reach the end of the G-less cassette will be termed "halted" and in either case, transcripts present in the supernatant will still be termed "released". Transcription complexes are assembled in extract from non-hydroxyurea-treated Wt-infected cells. Following a short pulse reaction, radiolabeled ternary complexes are isolated, washed and resuspended in a mixture containing nucleotides including 3'-OMeGTP and combinations of proteins as indicated (Fig. 22B). Ternary complexes reconstituted with only nucleotides do not recognize the early termination signal, transcribe and halt at the end of the G-less cassette and are not released into the supernatant (Fig. 22B, Lanes 1 and 2). In the presence of purified CE and NPH-I, 71% of the complexes "terminate" in response to the termination signal (Fig. 22B, *) and the remaining 29% are "halted" at the end of the G-less cassette (Fig. 22B, #, Lanes 3 and 4). Approximately 58% of the "terminated" transcripts and 52% of the halted transcripts are released into the supernatant. Ternary complexes elongated in the presence of His-A18 and CF (supplied by extract from mock-infected A549 cells) do not

Fig. 22. **CF has an effect on release of terminated transcripts.** *A*, diagram of the pSB24term template used for transcription. The DNA template (*line*) contains a biotinylated ATP incorporated at both the 5' and 3' ends, which anchors the DNA to a streptavidin-coated magnetic bead (*circles*). The bead is anchored 220 nt from the promoter at the 5' end of the template. The transcription unit consists of a synthetic early promoter (*arrow*) fused to a G-less cassette that is 540 nt in length. Within the G-less cassette is the early gene specific termination signal (*U5NU*) which, if recognized, will cause termination at approximately 450 nt downstream from the promoter. *B*, autoradiogram of the transcript release assay. Transcription complexes were generated in extract from non-hydroxyurea-treated Wt-infected cells. Isolated, washed ternary complexes were chased with the addition of 0.6 mM ATP and UTP, 1.2 mM CTP, and 0.01 mM 3'-OMeGTP (*NTPs*) or nucleotides plus purified vv capping enzyme (*CE*), purified vv NPH-I (*NPH-I*), His-A18(*A18*), and/or Mock extract (*Mock*) as indicated above the lanes. "Terminated" transcripts are indicated by the (*) and "halted" transcripts are indicated by the (#). *B*, bound; *S*, released.

A. pSB24term



B.



recognize the early termination signal but do halt at the end of the G-less cassette and, in this experiment, release approximately 24% of the transcripts (Fig. 22B, Lanes 5 and 6). Interestingly, the addition of His-A18, CF, CE, and NPH-I to ternary complexes did not significantly affect the recognition of the termination signal (63% "terminated" as compared to 71% in the presence of only CE and NPH-I) but did increase the release of the "terminated" transcripts (84% of "terminated" transcripts are released as compared to 58% release in the presence of only CE and NPH-I) (Fig. 22B, Lanes 7 and 8). In the absence of either CE or NPH-I, the termination signal is not recognized and release of the "halted" transcripts is dependent on His-A18 and CF (Fig. 22B, Lanes 9-12). The addition of only His-A18 protein to CE and NPH-I did not significantly change the release of "terminated" transcripts as compared to CE and NPH-I alone (Fig. 22B, compare Lanes 3 and 4 and Lanes 13 and 14). Surprisingly, the addition of CF to CE and NPH-I did increase release of the "terminated" transcripts (86% released with the addition of CF as compared to 58% release in the presence of only CE and NPH-I) but did not affect recognition of the termination signal (Fig. 22B, compare Lanes 3 and 4 and Lanes 15 and 16). Therefore, CF may enhance release of the early transcription "terminated" transcripts and may play a similar role in A18-dependent transcription.

CHAPTER 4
DISCUSSION

Previous research implicated the vaccinia virus *A18R*, *G2R*, and *J3R* gene products in the regulation of 3'-end formation of vaccinia virus intermediate stage transcripts. Specifically, mutations in the *G2R* and *J3R* genes result in the synthesis of 3' truncated transcripts (11,13,162) and mutations in the *A18R* gene result in synthesis of readthrough transcripts at intermediate times during infection (50). These results imply that the G2 and J3 proteins act as positive transcription elongation factors and the A18 protein acts as a negative transcription elongation factor. We developed immobilized template assays to study the effects of the A18, G2, and J3 proteins on elongation and release of nascent RNA from vaccinia virus transcription complexes. The results of this study allow us to draw several major conclusions. First, nascent transcript release requires A18 protein and an additional activity (cellular factor) that can be provided by either uninfected cell extract or extract from *A18R* mutant (Cts23)-infected cells. Second, the A18 protein and/or the cellular factor must be present during elongation in order for release to occur. Third, release requires a stalled transcription elongation complex. Fourth, transcript release requires ATP hydrolysis. Fifth, the cellular factor is not human Factor 2. Sixth, the cellular factor binds to anion exchange and hydroxyapatite resins but does not bind to cation exchange resin. Seventh, the transcript release activity in mock extract and purified A18 protein can catalyze release of transcripts synthesized from promoters representing all stages of vaccinia transcription. Finally, CF has an effect on

the release of the early transcription "terminated" transcripts and may play a similar role in A18-dependent transcription.

Transcript Release Requires A18 and a Cellular Factor

A18 protein alone cannot induce transcript release but requires an additional activity that is provided by extract from either uninfected or Cts23-infected cells. The activity is heat-labile as demonstrated by the abolishment of transcript release after heating the extract for 10 min at either 50°C or 65°C. The simplest explanation for these observations is that the additional activity required for A18-dependent release is a cellular factor(s). Another possible explanation is that the factor from Cts23-infected cells and the factor from uninfected cells are different. Therefore, the extract from mock-infected cells is providing an analogous activity or an activity that abolishes the need for the viral factor. The proof of either hypothesis requires that this factor be purified and identified from uninfected cell extract and potentially Cts23-infected cell extract.

The participation of cellular factors in vaccinia virus transcription is not without precedent. The intermediate transcription initiation factor, VITF-2, is provided by the nucleus of uninfected cells (132). The first identified cellular factor, YY-1. binds to a vaccinia promoter of the intermediate class and activates transcription *in vitro* (Steven Broyles, personal communication). Another cellular factor, VLTF-X, is an RNA binding protein and is required for late gene transcription initiation *in vitro* (Cynthia Wright, personal communication). Taking into account the fact that A18 and the cellular factor, CF, act on polymerase complexes initiated from all three stages of transcription, any of the previously mentioned cellular factors could be the activity we have discovered.

Mechanistic Requirements for Transcript Release

We have observed that the A18 protein and/or the cellular factor must be present during elongation in order for transcript release to occur. The factors need not be present during initiation, because washed elongation complexes that are incapable of transcript release can be induced to release RNA by the subsequent addition of A18 protein and CF. Whether both A18 and CF must be present during elongation remains to be determined. These results suggest that at least one of the two factors may become associated with the elongation complex after initiation and "ride" the complex, poised for arrest and termination. This phenomenon is not without precedent. For example, the eukaryotic elongation factors TFIIF (153), Elongin (7), and ELL (144) and the prokaryotic elongation factors GreA (13), Q (168), and N (128) must all form an association with their cognate elongation complex as a prerequisite to activity (155).

We have observed that the elongation complex must be stalled in order for transcript release to occur. Specifically, detection of transcript release *in vitro* requires a halted polymerase induced by inclusion of NaCl during the elongation reaction, by transcribing to a bead attached to the downstream end of a DNA template, or by transcribing to the end of a G-less cassette in the absence of GTP or in the presence of 3'-OMeGTP. We have tested elongation through both bacterial plasmid sequences and authentic viral sequences, and we have observed no effect of specific nucleic acid sequence on transcript release *in vitro*. *In vivo*, transcripts synthesized from either intermediate or late genes are heterogeneous in length, consistent with a sequence-independent termination event. The possibility exists that other factors *in vivo* may act to either pause or arrest the transcription complex prior to termination. Thus termination of post-replicative transcription in vaccinia may resemble murine RNAPI termination,

where transcription is blocked by TTF-I prior to transcript release catalyzed by PTRF (62,91).

The A18 protein shares a requirement for ATP hydrolysis with several transcription termination factors from both prokaryotic and eukaryotic systems (9,155). These factors include Rho (125), La (87), Factor 2 (164), and NPH-I (42). Each protein requires a different nucleic acid cofactor for its activity. The A18 ATPase activity is stimulated by single-stranded DNA, double-stranded DNA, and DNA-RNA hybrids, similar to NPH-I, Factor 2, and La, respectively. Of these termination factors, only Rho and A18 have identified helicase activity. The other proteins contain helicase motifs; however, no helicase activity has been described. The weak helicase activity of A18 is capable of unwinding a DNA duplex that is 20-nt or less (147). The protein is also capable of binding single-stranded DNA in the absence of ATP. Although our results demonstrate that transcript release is dependent upon ATP hydrolysis, this could be the activity of A18 or the unidentified cellular factor. To identify the ATP-dependent factor, we attempted to purify the *A18R* mutant protein (D206N) for analysis in the transcript release assay. This mutation results in a single amino acid change within the Walker B box sequence proposed to be associated with ATP binding and should have the effect of reducing the ATPase activity of A18. Unfortunately, this mutation destabilized the protein, preventing its purification and subsequent use in the transcript release assay. Based on these findings we propose that during elongation the A18 protein is recruited to the ternary complex by binding to the single-stranded non-template DNA strand in the region of the transcription bubble (157) and awaits activation of both ATPase and helicase activities to induce transcript release. This activity would be similar to that

proposed for NPH-I, the energy coupling factor required for vaccinia early gene transcription termination. NPH-I is postulated to bind to the non-template DNA strand within the transcription bubble. Recognition of the early termination signal by the vaccinia termination factor (CE/VTF) activates the ATPase activity of NPH-I resulting in the release of the nascent RNA (28,42).

Biochemical Characterization of the Cellular Factor

We hypothesized that human Factor 2 may substitute for CF in vaccinia transcript release for several reasons. First, human Factor 2 was identified based on its ability to release transcripts from RNAPII early elongation complexes. Factor 2 will release RNA from any RNAPII ternary complex *in vitro*. This activity closely resembles the A18-dependent transcript release we have identified in vaccinia virus. Second, Factor 2 is a strong DNA-dependent ATPase and possesses a helicase motif although helicase activity has not been detected (164). ATP hydrolysis is required for A18-dependent transcript release and the responsible factor could be either A18 or CF. Third, recent data indicates that Factor 2 is also able to disrupt RNAPII, as well as RNAPI, ternary complexes stalled at a thymine cyclobutane dimer (54). The discovery of a transcription elongation factor with activity on different classes of RNAP is not unprecedented. In addition to Factor 2, TFIIS has been shown to cause transcript cleavage during both RNAPI and RNAPII transcription elongation (137). However, our data indicate that in fact Factor 2 does not substitute for either A18 or CF and has no effect on A18-dependent release from a vaccinia virus promoter.

To purify CF we took clues from purification schemes for other identified proteins. Fortunately, the activity was present in uninfected HeLa cells, allowing

purification without large volumes of vaccinia-infected cells. We started fractionation with anion exchange and affinity chromatography that separates proteins based on charge or affinity, respectively. Multiple fractionation attempts using both types of resin has led to an unsatisfactory degree of purification characterized by a broad elution pattern suggesting several hypotheses. First, although the chromatography conditions are appropriate for the purification of the A18 protein (Figure 12), the conditions may not be optimal for purification of the cellular factor. The elution conditions, such as the substitution of phosphate for NaCl, could be altered to determine whether the activity elution pattern would be affected. Second, the broad elution pattern may be an indication that the cellular factor is composed of multiple proteins. In this case, the broad pattern is a result of the different binding capabilities of the multiple proteins. One experiment to address this question is the use of a glycerol gradient or a sizing column. In either case, the proteins present in a given fraction are separated based on their relative mass and could be tested individually in the *in vitro* assay. Third, the results could be due to poor chromatography methods. Fourth, the CF activity in transcript release may be a non-specific effect of the *in vitro* assay.

We addressed the possibility of poor chromatography by reexamining the results of the Wt extract partial purification in Fig. 12. Fractionation of the Wt extract resulted in the isolation of individual fractions containing both the A18 protein and transcript release activity as demonstrated by western blot analysis and the *in vitro* transcript release assay. Transcript release did not require the addition of mock extract, evidence that the cellular factor was present in the fractions. There are two possible hypotheses for the presence of CF in the fractions: CF may cofractionate with A18 due to intrinsic

properties or a direct interaction with A18, or CF may elute in all of the fractions, similar to the broad elution patterns obtained during purification attempts of CF from uninfected HCE. The latter hypothesis is supported by the analysis of partially purified fractions from Cts23 extract (data not shown) in a protocol identical to that used with the Wt extract. The Cts23 fractions were assayed for *in vitro* transcript release activity with the addition of His-A18 purified protein. The results showed that CF is present in almost all of the fractions from both the phosphocellulose and Q-Sepharose columns. The control for the chromatography conditions was provided by the Wt extract fractionation where A18 protein purified in a single elution from both columns. Therefore, it is likely that the broad elution of CF is a property of CF itself rather than a result of chromatography conditions. Although this experiment demonstrates the behavior of CF in an extract devoid of A18 protein, it does not directly address the possibility of an interaction between A18 and CF that would be highly useful for purification of CF. We therefore propose that the partially purified Wt extract fractions be reexamined for *in vitro* transcript release activity in combination with His-A18 purified protein. An interaction between A18 and CF would be shown by the presence of transcript release activity only in Wt extract fractions that contain endogenous A18 protein. If CF does not specifically interact with A18, transcript release activity would also be present in fractions that did not contain endogenous A18 protein. Although this experiment does not directly demonstrate an interaction between A18 and CF, the data would allow us to evaluate the potential for using A18 to purify CF.

An observation made by Landick (personal communication) indicated that the streptavidin-coated magnetic beads non-specifically bind negatively charged molecules

including nucleic acids and proteins. This suggests that the streptavidin molecules on the surface of the magnetic beads bind the RNA that is released when transcription elongation is performed in the presence of A18 protein alone thereby inhibiting release of the RNA into the supernatant. The addition of extract may only provide negatively-charged molecules to coat the streptavidin on the magnetic beads thereby preventing the interaction of streptavidin with released RNA. This effect would result in a requirement for extract, or a hypothesized cellular factor, in the *in vitro* assay. As a test of this hypothesis, tRNA was used in the transcript release assay in place of uninfected cell extract (in reactions containing His-A18) and no transcript release was detected (data not shown). Therefore, we have concluded that this observation is not relevant to our system. Additional experiments could include the assay of extract from either bacteria or uninfected insect cells. We might expect, due to the host range of vaccinia virus, that either of these sources would not contain a putative cellular factor necessary for vaccinia transcription and would therefore further test the hypothesis that the necessity of a cellular factor(s) is a nonspecific effect.

An additional observation was made during purification of the cellular factor when it revealed that CF is not retained on phosphocellulose during fractionation of uninfected HCE (Fig. 20). This is in contrast to the partial purification of Wt extract on phosphocellulose that resulted in one fraction, 0.5 M NaCl, which contained A18 protein and transcript release activity (Fig. 12). Further experiments to test for the presence of CF in the other salt elutions are proposed above. Partial purification of Cts23 extract showed release activity in almost all of the phosphocellulose fractions when supplemented with purified A18 protein. The difference between the HCE fractionation

and the fractionations of Wt and Cts23 extract is the absence or presence of viral proteins. Based on the different chromatographic properties, one hypothesis is that the viral and cellular transcript release activities are in fact different. Another hypothesis is that the cellular factor interacts with viral proteins in the Wt or Cts23 extract that enables the retention of CF on phosphocellulose. In the absence of those viral proteins, as in HCE, the activity does not bind phosphocellulose. It is not clear, in the absence of the proposed experiments, whether A18 is responsible for the retention of CF on phosphocellulose when purified from Wt or Cts23 extract or, if this is an indication that the factor in extract of uninfected and infected cells is different.

Role of A18/CF-Dependent Release Throughout Infection

The observation that the A18 protein catalyzes transcript release from early as well as late transcription complexes suggests that A18 acts on all three classes of transcription *in vivo*. Early transcription complexes are significantly different than intermediate and late transcription complexes both in structure and function (98). Early transcription is catalyzed entirely by enzymes packaged in the virion and presumably occurs within uncoated viral core particles *in vivo*. By contrast, intermediate and late transcription occurs in viral DNA-containing cytoplasmic centers of replication called virosomes. Early complexes contain RAP94, a viral factor that is required for early transcription initiation that remains strongly associated with the early elongation complex, while intermediate and late transcription complexes probably lack this factor. Early transcripts are homogeneous in length, resulting from recognition of a cis-acting RNA sequence by the vaccinia virus capping enzyme and NPH-I, whereas intermediate and late transcripts are heterogeneous in length. Nevertheless, the fact that the A18

protein is synthesized throughout infection and packaged within virions supports a role for A18 during early transcription. In addition, recent data indicates that A18 interacts with J3 as well as NPH-I (Edward Niles, personal communication). Both J3 and NPH-I have roles in early transcription as a capping and polyadenylation factor and for recognition of the early transcription termination signal, respectively. The *A18R* mutations do not affect early viral transcription *in vivo* (8), but this is not untypical for mutations in vaccinia virion enzymes. For example, temperature-sensitive mutants in the virion early transcription initiation factor VETF (27), the RNA helicase NPH-2 (44), the mRNA capping enzyme (55), and the RNA polymerase (60,61) have shown no pronounced effect on early transcription *in vivo*. Although the mechanism of early transcription termination is reasonably well understood, a role for A18 as an early transcript release factor has not been ruled out.

Although cellular factor activity has been characterized in vaccinia virus intermediate and late transcription, host cell activity has not been previously described for early transcription. The observations that CF has an effect on transcript release of vaccinia virus early "terminated" transcripts and transcripts released in an A18-dependent manner and the presumption that early transcription is catalyzed by enzymes packaged in the virion and occurs within the uncoated viral core, suggest that the cellular factor is packaged within the virion. This could be of potential use for the purification of the cellular factor. Although this does not eliminate the many viral proteins that are packaged, there are significantly fewer proteins present in virions as compared to either an infected or an uninfected cell.

What is the role of a cellular factor in early transcription? The identification of cellular factors required for intermediate and late transcription suggested a mechanism where the virus could "sense" the suitability of the cell environment for production of progeny virions. The cellular factor identified in this study appears to be abundant, as it is present in both the cytoplasm and nucleus of uninfected cells and its role in transcript release at all three classes of vaccinia virus promoters suggests that it is abundant in virosomes as well. One hypothesis is that the virus has recruited the cellular factor for a specific role in vaccinia transcript release and conserves that activity by packaging CF for the same function in early transcription. On the other hand, the role of the cellular factor in early transcription may be a product of the *in vitro* release assay. The cellular factor may not be packaged in virions but the inherent activity of CF could still function on an early complex *in vitro*. This suggests a model for A18-dependent termination that is neither sequence- nor promoter-specific. Both A18 and CF are recruited to ternary complexes to terminate transcription and release the polymerase and nascent RNA. The presence of positive elongation factors may either prevent interaction of A18 and CF with the complex or prevent their action *in vivo*. A18 and CF only act when transcription is no longer feasible, such as in the presence of high salt or 3'-OMeGTP *in vitro* or when positive elongation factors cannot function *in vivo*. CF can release early transcripts terminated by NPH-I and VTF suggesting that the role of CF may be to release transcripts that have been terminated by any mechanism.

Future Directions

We hypothesize that the G2 and J3 proteins function as positive transcription elongation factors. Preliminary results using limited quantities of either protein did not

show any activity in the elongation assay. One explanation is that the reaction conditions are not optimal for elucidation of the G2 and J3 phenotypes. These proteins were not thoroughly exploited once the release assay and a phenotype for A18 were discovered. Further manipulations of the elongation assay including titration of both G2 and J3 and combinations of purified proteins, including G2, J3, A18, and H5, and/or Wt and mutant extract should be further evaluated for effects on both elongation or termination.

The inclusion of NaCl during the elongation assay (Fig. 7) resulted in increased pausing of the ternary complex and has potential as an assay for characterization of the positive elongation factors. This assay has two major differences from the mid-template transcript release assay. First, the pulse-labeled elongation complexes were washed with low salt buffer, resulting in a potentially less pure ternary complex. Second, the elongation assay was performed in the presence of Wt extract and not purified A18 protein and uninfected HCE. It is possible that the NaCl effect on elongation (Fig. 7) is due to positive elongation factors that are associated with the less stringently washed complexes that are then removed when NaCl is included in the elongation reaction. Using protocols that include either the low salt or high salt wash conditions, ternary complexes formed in either Wt or mutant extract (lacking either G2 or J3) should be compared for transcription elongation and release in the presence of NaCl and Wt extract, Cts23 and A18 protein, or HCE and A18 protein to determine whether NaCl has a similar effect on complexes that may contain or lack the putative positive elongation factors. Formation of transcription complexes lacking either G2 or J3 may have a similar phenotype. However, we suspect that the presence of J3, for example, in the G2 mutant extract may be able to compensate for the lack of G2. In this case the elongation

phenotype of the NaCl washed complexes may still differ from either G2 or J3 mutant complexes. The stringent high salt wash buffer removes nonspecifically bound proteins that, in the past, have interfered with the release assay. However, it is possible that the positive elongation factors are not tightly associated with the complex and are removed with the high salt wash. These factors may not reassociate with a ternary complex so addition of the proteins during elongation may not have an effect. Alternatively, the NaCl may prevent the reassociation of the factors with the complex. Therefore, comparison of the elongation phenotype in the presence (Wt extract) and absence (A18 and HCE) of unknown viral proteins using complexes which have been washed in sufficiently high salt may indicate whether there are positive elongation factors that can be elucidated using this assay. Further experimentation using similar assays should then be performed using purified G2, H5, and J3 proteins in combination with A18 and HCE to determine whether these proteins are contributing to the positive elongation activity.

Summary

Genetic and biochemical evidence suggests that both vaccinia intermediate and late gene transcription elongation are regulated by several viral genes in addition to *A18R*. The fact that both intermediate and late transcripts possess heterogeneous 3'-ends implies a similar mechanism of transcription termination for both gene classes. Mutation of either gene *G2R* (11) or *J3R* (162) results in synthesis of 3'-truncated intermediate and late viral mRNAs, implying that each of these gene products exerts positive transcription elongation factor activity on both intermediate and late viral genes. Mutations in either *G2R* (35) or *J3R* (78) suppress *A18R* mutations, strongly suggesting that all three genes function in the same pathway. Interestingly, the *J3R* gene product was previously shown

to encode a protein with both 2'-O-methyltransferase and poly(A) polymerase processivity activities (138); no distinct biochemical activity has yet been identified for the G2 protein (12). One additional protein, the viral *H5R* gene product, was shown to associate directly with the G2 protein, suggesting that these two proteins may both be involved in transcription elongation (12). Finally, evidence exists that the A18, G2, and H5 proteins are all associated either directly or indirectly as a complex *in vivo* (12).

In summary, the evidence to date suggests that intermediate and late gene transcription elongation in vaccinia is controlled by a complex of viral and cellular factors possessing both positive and negative elongation factor activities. The powerful combination of genetics and the *in vitro* system described here provide us with an opportunity to investigate the activities of the individual components of this transcription elongation complex. Vaccinia has often served as a valuable model system for transcription in higher eukaryotes, and thus these studies of vaccinia transcription elongation and termination may provide insight into the same processes in mammalian cells.

APPENDIX
TABLE OF ABBREVIATIONS

WT	wild type
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid
RNAP	RNA polymerase
Taq	*Thermus aquaticus*
rRNA	ribosomal RNA
tRNA	transfer RNA
GTFs	general transcription factors
CTD	carboxy-terminal domain
P-TEF	positive transcription elongation factor
E. coli	*Escherichia coli*
S. cerevisiae	*Sacchromyces cerevisiae*
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
kDa	kiloDalton
Srb	suppressors of RNA polymerase II
MEDs	mediator proteins
PIC	preinitiation complex
TBP	TATA binding protein
RSC	remodels the structure of chromatin
CHRAC	chromatin accessibility complex
RSF	remodeling and spacing factor
ACF	ATP-utilizing chromatin assembly and remodeling factor
Inr	initiator
ATP	adenosine triphosphate
CTP	cytosine triphosphate
GTP	guanine triphosphate
UTP	uracil triphosphate
nt	nucleotide
ATPase	adenosine triphosphatase
N-TEF	negative transcription elongation factor
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
NELF	negative elongation factor
DSIF	DRB-sensitivity inducing factor
Cdk	cyclin-dependent kinase
Mg	magnesium
bp	base pairs
FACT	facilitates chromatin transcription

PTRF	RNA polymerase I transcript release factor
TTF-1	Transcription termination factor for RNA polymerase I
VHL	von Hippel-Lindau
VTF	vaccinia termination factor
CE	capping enzyme
NPH-I	nucleoside triphosphate phosphohydrolase-I
VETF	vaccinia early transcription factor
VITF	vaccinia intermediate transcription factor
VLTF	vaccinia late transcription factor
YY1	ying-yang protein 1
ts	temperature-sensitive
RNase	ribonuclease
RT-PCR	reverse transcriptase polymerase chain reaction
IBT	isatin-β-thiosemicarbazone
NaCl	sodium chloride
NTPs	nucleoside triphosphates
CF	cellular factor
HNE	HeLa nuclear extract
HCE	HeLa cytoplasmic extract
3'OMeGTP	3'O methyl GTP
vv	vaccinia virus
Fig	figure

REFERENCES

1. **Ahn, B. Y., P. D. Gershon, E. V. Jones, and B. Moss**. 1990. Identification of rpo30, a vaccinia virus RNA polymerase gene with structural similarity to a eucaryotic transcription elongation factor. Mol.Cell Biol. **10**:5433-5441.

2. **Ahn, B. Y. and B. Moss**. 1989. Capped poly(A) leaders of variable lengths at the 5' ends of vaccinia virus late mRNAs. J.Virol. **63**:226-232.

3. **Arndt, K. M. and M. J. Chamberlin**. 1990. RNA chain elongation by Escherichia coli RNA polymerase. Factors affecting the stability of elongating ternary complexes. J.Mol.Biol. **213**:79-108.

4. **Artsimovitch, I. and R. Landick** . 1998. Interaction of a nascent RNA structure with RNA polymerase is required for hairpin-dependent transcriptional pausing but not for transcript release. Genes Dev. **12**:3110-3122.

5. **Artsimovitch, I. and R. Landick** . 2000. Pausing by bacterial RNA polymerase is mediated by mechanistically distinct classes of signals. Proc.Natl.Acad.Sci.U.S.A **97**:7090-7095.

6. **Aso, T., D. Haque, R. J. Barstead, R. C. Conaway, and J. W. Conaway**. 1996. The inducible elongin A elongation activation domain: structure, function and interaction with the elongin BC complex. EMBO J. **15**:5557-5566.

7. **Aso, T., W. S. Lane, J. W. Conaway, and R. C. Conaway**. 1995. Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II [see comments]. Science **269**:1439-1443.

8. **Bayliss, C. D. and R. C. Condit** . 1993. Temperature-sensitive mutants in the vaccinia virus A18R gene increase double-stranded RNA synthesis as a result of aberrant viral transcription. Virology **194**:254-262.

9. **Bayliss, C. D. and R. C. Condit** . 1995. The vaccinia virus A18R gene product is a DNA-dependent ATPase. J.Biol.Chem. **270**:1550-1556.

10. **Beaud, G. and R. Beaud**. 1997. Preferential virosomal location of underphosphorylated H5R protein synthesized in vaccinia virus-infected cells. J.Gen.Virol. **78 (Pt 12)**:3297-3302.

11. **Black, E. P. and R. C. Condit**. 1996. Phenotypic characterization of mutants in vaccinia virus gene G2R, a putative transcription elongation factor. J.Virol. **70**:47-54.

12. **Black, E. P., N. Moussatche, and R. C. Condit**. 1998. Characterization of the interactions among vaccinia virus transcription factors G2R, A18R, and H5R. Virology **245**:313-322.

13. **Borukhov, S., V. Sagitov, and A. Goldfarb**. 1993. Transcript cleavage factors from E. coli. Cell **72**:459-466.

14. **Brown, S. A., A. N. Imbalzano, and R. E. Kingston**. 1996. Activator-dependent regulation of transcriptional pausing on nucleosomal templates. Genes Dev. **10**:1479-1490.

15. **Broyles, S. S.** 1991. A role for ATP hydrolysis in vaccinia virus early gene transcription. Dissociation of the early transcription factor-promoter complex. J.Biol.Chem. **266**:15545-15548.

16. **Broyles, S. S. and B. S. Fesler**. 1990. Vaccinia virus gene encoding a component of the viral early transcription factor. J.Virol. **64**:1523-1529.

17. **Broyles, S. S., X. Liu, M. Zhu, and M. Kremer**. 1999. Transcription factor YY1 is a vaccinia virus late promoter activator. J.Biol.Chem. **274**:35662-35667.

18. **Broyles, S. S. and B. Moss**. 1988. DNA-dependent ATPase activity associated with vaccinia virus early transcription factor. J.Biol.Chem. **263**:10761-10765.

19. **Burgess, R. R., A. A. Travers, J. J. Dunn, and E. K. Bautz**. 1969. Factor stimulating transcription by RNA polymerase. Nature **221**:43-46.

20. **Cairns, B. R.** 1998. Chromatin remodeling machines: similar motors, ulterior motives. Trends Biochem.Sci. **23**:20-25.

21. **Chamberlin, M. J**. 1995 New Models for the Mechanism of Transcription Elongation and its Regulation. Harvey Lectures **88**: 1-21.

22. **Chan, C. L. and R. Landick**. 1994. New Perspectives on RNA Chain Elongation and Termination by *E. coli* RNA polymerase, p. 297-321. *In* R. C. Conaway and J. W. Conaway (ed.), Transcription: Mechanisms and Regulation. Raven Press, Ltd., New York.

23. **Chan, C. L. and R. Landick**. 1993. Dissection of the his leader pause site by base substitution reveals a multipartite signal that includes a pause RNA hairpin. J.Mol.Biol. **233**:25-42.

24. **Chan, C. L., D. Wang, and R. Landick**. 1997. Multiple interactions stabilize a single paused transcription intermediate in which hairpin to 3' end spacing distinguishes pause and termination pathways. J.Mol.Biol. **268**:54-68.

25. **Cho, H., G. Orphanides, X. Sun, X. J. Yang, V. Ogryzko, E. Lees, Y. Nakatani, and D. Reinberg**. 1998. A human RNA polymerase II complex containing factors that modify chromatin structure. Mol.Cell Biol. **18**:5355-5363.

26. **Chodosh, L. A., A. Fire, M. Samuels, and P. A. Sharp**. 1989. 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. J.Biol.Chem. **264**:2250-2257.

27. **Christen, L., M. A. Higman, and E. G. Niles**. 1992. Phenotypic characterization of three temperature-sensitive mutations in the vaccinia virus early gene transcription initiation factor. J.Gen.Virol. **73 (Pt 12)**:3155-3167.

28. **Christen, L. M., M. Sanders, C. Wiler, and E. G. Niles**. 1998. Vaccinia virus nucleoside triphosphate phosphohydrolase I is an essential viral early gene transcription termination factor. Virology **245**:360-371.

29. **Citron, B., E. Falck-Pedersen, M. Salditt-Georgieff, and J. E. Darnell, Jr.** 1984. Transcription termination occurs within a 1000 base pair region downstream from the poly(A) site of the mouse beta-globin (major) gene. Nucleic Acids Res. **12**:8723-8731.

30. **Coleman, R. A., A. K. Taggart, S. Burma, J. J. Chicca, and B. F. Pugh**. 1999. TFIIA regulates TBP and TFIID dimers. Mol.Cell **4**:451-457.

31. **Colinas, R. J., R. C. Condit, and E. Paoletti**. 1990. Extrachromosomal recombination in vaccinia-infected cells requires a functional DNA polymerase participating at a level other than DNA replication. Virus Res. **18**:49-70.

32. **Condit, R. C., J. I. Lewis, M. Quinn, L. M. Christen, and E. G. Niles**. 1996. Use of lysolecithin-permeabilized infected-cell extracts to investigate the in vitro biochemical phenotypes of poxvirus ts mutations altered in viral transcription activity. Virology **218**:169-180.

33. **Condit, R. C. and A. Motyczka**. 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology **113**:224-241.

34. **Condit, R. C., A. Motyczka, and G. Spizz**. 1983. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. Virology **128**:429-443.

35. **Condit, R. C., Y. Xiang, and J. I. Lewis**. 1996. Mutation of vaccinia virus gene G2R causes suppression of gene A18R ts mutants: implications for control of transcription. Virology **220**:10-19.

36. **Darst, S. A., E. W. Kubalek, and R. D. Kornberg**. 1989. Three-dimensional structure of Escherichia coli RNA polymerase holoenzyme determined by electron crystallography. Nature **340**:730-732.

37. **Darst, S. A., A. Polyakov, C. Richter, and G. Zhang**. 1998. Insights into Escherichia coli RNA polymerase structure from a combination of x-ray and electron crystallography. J.Struct.Biol. **124**:115-122.

38. **Das, A.** 1993. Control of transcription termination by RNA-binding proteins. Annu.Rev.Biochem. **62**:893-930.

39. **Davenport, R. J., G. J. Wuite, R. Landick, and C. Bustamante**. 2000. Single-molecule study of transcriptional pausing and arrest by E. coli RNA polymerase [see comments]. Science **287**:2497-2500.

40. **deHaseth, P. L., M. L. Zupancic, and M. T. Record, Jr.** 1998. RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. J.Bacteriol. **180**:3019-3025.

41. **Deng, L., J. Hagler, and S. Shuman**. 1996. Factor-dependent release of nascent RNA by ternary complexes of vaccinia RNA polymerase. J.Biol.Chem. **271**:19556-19562.

42. **Deng, L. and S. Shuman**. 1998. Vaccinia NPH-I, a DExH-box ATPase, is the energy coupling factor for mRNA transcription termination. Genes Dev. **12**:538-546.

43. **Dikstein, R., S. Ruppert, and R. Tjian**. 1996. TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. Cell **84**:781-790.

44. **Fathi, Z. and R. C. Condit**. 1991. Phenotypic characterization of a vaccinia virus temperature-sensitive complementation group affecting a virion component. Virology **181**:273-276.

45. **Flanagan, P. M., R. J. Kelleher, III, M. H. Sayre, H. Tschochner, and R. D. Kornberg**. 1991. A mediator required for activation of RNA polymerase II transcription in vitro. Nature **350**:436-438.

46. **Gamper, H. B. and J. E. Hearst** . 1982. A topological model for transcription based on unwinding angle analysis of E. coli RNA polymerase binary, initiation and ternary complexes. Cell **29**:81-90.

47. **Garrett, K. P., T. Aso, J. N. Bradsher, S. I. Foundling, W. S. Lane, R. C. Conaway, and J. W. Conaway**. 1995. Positive regulation of general transcription factor SIII by a tailed ubiquitin homolog. Proc.Natl.Acad.Sci.U.S.A **92**:7172-7176.

48. **Golini, F. and J. R. Kates**. 1985. A soluble transcription system derived from purified vaccinia virions. J.Virol. **53**:205-213.

49. **Gu, W., M. Wind, and D. Reines** . 1996. Increased accommodation of nascent RNA in a product site on RNA polymerase II during arrest. Proc.Natl.Acad.Sci.U.S.A **93**:6935-6940.

50. **Gunasinghe, S. K., A. E. Hubbs, and C. F. Wright**. 1998. A vaccinia virus late transcription factor with biochemical and molecular identity to a human cellular protein. J.Biol.Chem. **273**:27524-27530.

51. **Hagenbuchle, O., P. K. Wellauer, D. L. Cribbs, and U. Schibler**. 1984. Termination of transcription in the mouse alpha-amylase gene Amy-2a occurs at multiple sites downstream of the polyadenylation site. Cell **38**:737-744.

52. **Hagler, J., Y. Luo, and S. Shuman**. 1994. Factor-dependent transcription termination by vaccinia RNA polymerase. Kinetic coupling and requirement for ATP hydrolysis. J.Biol.Chem. **269**:10050-10060.

53. **Han, M. and M. Grunstein**. 1988. Nucleosome loss activates yeast downstream promoters in vivo. Cell **55**:1137-1145.

54. **Hara, R., C. P. Selby, M. Liu, D. H. Price, and A. Sancar**. 1999. Human transcription release factor 2 dissociates RNA polymerases I and II stalled at a cyclobutane thymine dimer. J.Biol.Chem. **274**:24779-24786.

55. **Hassett, D. E., J. I. Lewis, X. Xing, L. DeLange, and R. C. Condit**. 1997. Analysis of a temperature-sensitive vaccinia virus mutant in the viral mRNA capping enzyme isolated by clustered charge-to-alanine mutagenesis and transient dominant selection. Virology **238**:391-409.

56. **Helmann, J. D.** 1991. Alternative sigma factors and the regulation of flagellar gene expression. Mol.Microbiol. **5**:2875-2882.

57. **Helmann, J. D. and M. J. Chamberlin**. 1988. Structure and function of bacterial sigma factors. Annu.Rev.Biochem. **57**:839-872.

58. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of rpoS in early stationary phase gene regulation in E. coli. Cell **72**:165-168.

59. **Holstege, F. C., U. Fiedler, and H. T. Timmers**. 1997. Three transitions in the RNA polymerase II transcription complex during initiation. EMBO J. **16**:7468-7480.

60. **Hooda-Dhingra, U., D. D. Patel, D. J. Pickup, and R. C. Condit**. 1990. Fine structure mapping and phenotypic analysis of five temperature-sensitive mutations in the second largest subunit of vaccinia virus DNA-dependent RNA polymerase. Virology **174**:60-69.

61. **Hooda-Dhingra, U., C. L. Thompson, and R. C. Condit**. 1989. Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J.Virol. **63**:714-729.

62. **Jansa, P., S. W. Mason, U. Hoffmann-Rohrer, and I. Grummt**. 1998. Cloning and functional characterization of PTRF, a novel protein which induces dissociation of paused ternary transcription complexes. EMBO J. **17**:2855-2864.

63. **Kashlev, M., E. Nudler, A. Goldfarb, T. White, and E. Kutter**. 1993. Bacteriophage T4 Alc protein: a transcription termination factor sensing local modification of DNA. Cell **75**:147-154.

64. **Keck, J. G., F. Feigenbaum, and B. Moss**. 1993. Mutational analysis of a predicted zinc-binding motif in the 26-kilodalton protein encoded by the vaccinia virus A2L gene: correlation of zinc binding with late transcriptional transactivation activity. J.Virol. **67**:5749-5753.

65. **Keck, J. G., G. R. Kovacs, and B. Moss**. 1993. Overexpression, purification, and late transcription factor activity of the 17-kilodalton protein encoded by the vaccinia virus A1L gene. J.Virol. **67**:5740-5748.

66. **Keene, R. G. and D. S. Luse**. 1999. Initially transcribed sequences strongly affect the extent of abortive initiation by RNA polymerase II. J.Biol.Chem. **274**:11526-11534.

67. **Kephart, D. D., B. Q. Wang, Z. F. Burton, and D. H. Price**. 1994. Functional analysis of Drosophila factor 5 (TFIIF), a general transcription factor. J.Biol.Chem. **269**:13536-13543.

68. **Kibel, A., O. Iliopoulos, J. A. DeCaprio, and W. G. Kaelin, Jr.** 1995. Binding of the von Hippel-Lindau tumor suppressor protein to Elongin B and C [see comments]. Science **269**:1444-1446.

69. **Komissarova, N. and M. Kashlev** . 1997. RNA polymerase switches between inactivated and activated states By translocating back and forth along the DNA and the RNA. J.Biol.Chem. **272**:15329-15338.

70. **Kovacs, G. R. and B. Moss**. 1996. The vaccinia virus H5R gene encodes late gene transcription factor 4: purification, cloning, and overexpression. J.Virol. **70**:6796-6802.

71. **Kovacs, G. R., R. Rosales, J. G. Keck, and B. Moss**. 1994. Modification of the cascade model for regulation of vaccinia virus gene expression: purification of a prereplicative, late-stage-specific transcription factor. J.Virol. **68**:3443-3447.

72. **Krakow, J. S., K. Daley, and M. Karstadt**. 1969. Azotobacter vinelandii RNA polymerase. VII. Enzyme transitions during unprimed r[I-C] synthesis. Proc.Natl.Acad.Sci.U.S.A **62**:432-437.

73. **Krummel, B. and M. J. Chamberlin**. 1992. Structural analysis of ternary complexes of Escherichia coli RNA polymerase. Individual complexes halted along different transcription units have distinct and unexpected biochemical properties. J.Mol.Biol. **225**:221-237.

74. **Kuras, L. and K. Struhl**. 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. Nature **399**:609-613.

75. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss**. 1989. Expression of sigma 54 (ntrA)-dependent genes is probably united by a common mechanism. Microbiol.Rev. **53**:367-376.

76. **Lackner CA and R. C. Condit.** 2000. Vaccinia virus gene A18R DNA helicase is a transcript release factor. J.Biol.Chem. **275**:1485-1494.

77. **Latif, F., K. Tory, J. Gnarra, M. Yao, F. M. Duh, M. L. Orcutt, T. Stackhouse, I. Kuzmin, W. Modi, and L. Geil** . 1993. Identification of the von Hippel-Lindau disease tumor suppressor gene [see comments]. Science **260**:1317-1320.

78. **Latner D. R., Y. Xiang, J. I. Lewis, J. Condit, R. C. Condit.** 2000. The Vaccinia Virus Bifunctional Gene J3 (Nucleoside-2'-O-)-methyltransferase and Poly(A) Polymerase Stimulatory Factor Is Implicated as a Positive Transcription Elongation Factor by Two Genetic Approaches. Virology. **269**:345-355.

79. **LeRoy, G., A. Loyola, W. S. Lane, and D. Reinberg**. 2000. Purification and characterization of a human factor that assembles and remodels chromatin. J.Biol.Chem. **275** :14787-14790.

80. **LeRoy, G., G. Orphanides, W. S. Lane, and D. Reinberg**. 1998. Requirement of RSF and FACT for transcription of chromatin templates in vitro [see comments]. Science **282**:1900-1904.

81. **Leuther, K. K., D. A. Bushnell, and R. D. Kornberg**. 1996. Two-dimensional crystallography of T. Cell **85**:773-779.

82. **Levin, J. R. and M. J. Chamberlin**. 1987. Mapping and characterization of transcriptional pause sites in the early genetic region of bacteriophage T7. J.Mol.Biol. **196**:61-84.

83. **Li, J. and S. S. Broyles**. 1993. The DNA-dependent ATPase activity of vaccinia virus early gene transcription factor is essential for its transcription activation function. J.Biol.Chem. **268**:20016-20021.

84. **Liu, M., Z. Xie, and D. H. Price**. 1998. A human RNA polymerase II transcription termination factor is a SWI2/SNF2 family member. J.Biol.Chem. **273**:25541-25544.

85. **Luo, Y., J. Hagler, and S. Shuman**. 1991. Discrete functional stages of vaccinia virus early transcription during a single round of RNA synthesis in vitro. J.Biol.Chem. **266**:13303-13310.

86. **Maldonado, E., R. Shiekhattar, M. Sheldon, H. Cho, R. Drapkin, P. Rickert, E. Lees, C. W. Anderson, S. Linn, and D. Reinberg**. 1996. A human RNA polymerase II complex associated with SRB and DNA-repair proteins [published erratum appears in Nature 1996 Nov 28;384(6607):384]. Nature **381**:86-89.

87. **Maraia, R. J.** 1996. Transcription termination factor La is also an initiation factor for RNA polymerase III. Proc.Natl.Acad.Sci.U.S.A **93**:3383-3387.

88. **Marshall, N. F., J. Peng, Z. Xie, and D. H. Price**. 1996. Control of RNA polymerase II elongation potential by a novel carboxyl- terminal domain kinase. J.Biol.Chem. **271**:27176-27183.

89. **Marshall, N. F. and D. H. Price**. 1992. Control of formation of two distinct classes of RNA polymerase II elongation complexes. Mol.Cell Biol. **12**:2078-2090.

90. **Marshall, N. F. and D. H. Price**. 1995. Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J.Biol.Chem. **270**:12335-12338.

91. **Mason, S. W., E. E. Sander, and I. Grummt**. 1997. Identification of a transcript release activity acting on ternary transcription complexes containing murine RNA polymerase I. EMBO J. **16**:163-172.

92. **McCraith, S., T. Holtzman, B. Moss, and S. Fields**. 2000. Genome-wide analysis of vaccinia virus protein-protein interactions. Proc.Natl.Acad.Sci.U.S.A **97**:4879-4884.

93. **Meis, R. J. and R. C. Condit**. 1991. Genetic and molecular biological characterization of a vaccinia virus gene which renders the virus dependent on isatin-beta-thiosemicarbazone (IBT). Virology **182**:442-454.

94. **Mohamed, M. R. and E. G. Niles**. 2000. Interaction between nucleoside triphosphate phosphohydrolase I and the H4L subunit of the viral RNA polymerase is required for vaccinia virus early gene transcript release. J.Biol.Chem. **275**:25798-25804.

95. **Mooney, R. A., I. Artsimovitch, and R. Landick**. 1998. Information processing by RNA polymerase: recognition of regulatory signals during RNA chain elongation. J.Bacteriol. **180**:3265-3275.

96. **Moss, B.** 1994. Vaccinia Virus Transcription, p. 185-205. *In* R. C. Conaway and J. W. Conaway (ed.), Transcription: Mechanisms and Regulation. Raven Press, Ltd., New York.

97. **Moss, B.** 1990. Regulation of vaccinia virus transcription. Annu.Rev.Biochem. **59**:661-688.

98. **Moss, B.** 1996. Poxviridae: The Viruses and Their Replication, p. 1163-1197. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fundamental Virology. Lippincott-Raven, Philadelphia.

99. **Moss, B., B. Y. Ahn, B. Amegadzie, P. D. Gershon, and J. G. Keck**. 1991. Cytoplasmic transcription system encoded by vaccinia virus. J.Biol.Chem. **266**:1355-1358.

100. **Moss, B., E. N. Rosenblum, and A. Gershowitz**. 1975. Characterization of a polyriboadenylate polymerase from vaccinia virions. J.Biol.Chem. **250**:4722-4729.

101. **Myer, V. E. and R. A. Young**. 1998. RNA polymerase II holoenzymes and subcomplexes. J.Biol.Chem. **273**:27757-27760.

102. **Myers, L. C., C. M. Gustafsson, D. A. Bushnell, M. Lui, H. Erdjument-Bromage, P. Tempst, and R. D. Kornberg**. 1998. The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. Genes Dev. **12**:45-54.

103. **Nevins, J. R. and W. K. Joklik**. 1977. Isolation and partial characterization of the poly(A) polymerases from HeLa cells infected with vaccinia virus. J.Biol.Chem. **252**:6939-6947.

104. **Nikolov, D. B., H. Chen, E. D. Halay, A. A. Usheva, K. Hisatake, D. K. Lee, R. G. Roeder, and S. K. Burley**. 1995. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. Nature **377**:119-128.

105. **Nonet, M. L. and R. A. Young**. 1989. Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of Saccharomyces cerevisiae RNA polymerase II. Genetics **123**:715-724.

105. **Novina, C. D. and A. L. Roy**. 1996. Core promoters and transcriptional control. Trends Genet. **12**:351-355.

106. **Nudler, E.** 1999. Transcription elongation: structural basis and mechanisms. J.Mol.Biol. **288**:1-12.

107. **Nudler, E., E. Avetissova, V. Markovtsov, and A. Goldfarb**. 1996. Transcription processivity: protein-DNA interactions holding together the elongation complex [see comments]. Science **273**:211-217.

108. **Nudler, E., A. Mustaev, E. Lukhtanov, and A. Goldfarb**. 1997. The RNA-DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. Cell **89**:33-41.

109. **Orlova, M., J. Newlands, A. Das, A. Goldfarb, and S. Borukhov**. 1995. Intrinsic transcript cleavage activity of RNA polymerase. Proc.Natl.Acad.Sci.U.S.A **92**:4596-4600.

110. **Orphanides, G., T. Lagrange, and D. Reinberg**. 1996. The general transcription factors of RNA polymerase II. Genes Dev. **10**:2657-2683.

111. **Orphanides, G., G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg**. 1998. FACT, a factor that facilitates transcript elongation through nucleosomes. Cell **92**:105-116.

112. **Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey, and D. Reinberg**. 1999. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. Nature **400**:284-288.

113. **Otero, G., J. Fellows, Y. Li, T. de Bizemont, A. M. Dirac, C. M. Gustafsson, H. Erdjument-Bromage, P. Tempst, and J. Q. Svejstrup**. 1999. Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. Mol.Cell **3**:109-118.

114. **Pacha, R. F. and R. C. Condit** . 1985. Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. J.Virol. **56**:395-403.

115. **Patel, D. D. and D. J. Pickup** . 1987. Messenger RNAs of a strongly-expressed late gene of cowpox virus contain 5'-terminal poly(A) sequences. EMBO J. **6**:3787-3794.

116. **Peng, J., M. Liu, J. Marion, Y. Zhu, and D. H. Price**. 1998. RNA polymerase II elongation control. Cold Spring Harb.Symp.Quant.Biol. **63**:365-370.

117. **Peng, J., N. F. Marshall, and D. H. Price**. 1998. Identification of a cyclin subunit required for the function of Drosophila P-TEFb. J.Biol.Chem. **273**:13855-13860.

118. **Peng, J., Y. Zhu, J. T. Milton, and D. H. Price**. 1998. Identification of multiple cyclin subunits of human P-TEFb. Genes Dev. **12**:755-762.

119. **Price, D. H.** 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol.Cell Biol. **20**:2629-2634.

120. **Proudfoot, N. J.** 1989. How RNA polymerase II terminates transcription in higher eukaryotes. Trends Biochem.Sci. **14**:105-110.

121. **Ranish, J. A., N. Yudkovsky, and S. Hahn**. 1999. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. Genes Dev. **13**:49-63.

122. **Reeder, R. H. and W. H. Lang**. 1997. Terminating transcription in eukaryotes: lessons learned from RNA polymerase I. Trends.Biochem.Sci. **22**:473-477.

123. **Rice, G. A., M. J. Chamberlin, and C. M. Kane**. 1993. Contacts between mammalian RNA polymerase II and the template DNA in a ternary elongation complex. Nucleic Acids Res. **21**:113-118.

124. **Richardson, J. P.** 1990. Rho-dependent transcription termination. Biochim.Biophys.Acta. **1048**:127-138.

125. **Richardson, J. P. and J. Greenblatt**. 1996, p. 822-848. *In* F. Neidhardt and et al (ed.), *E. coli* and *Salmonella*: Cellular and Molecular Biology. ASM Press, Washington D.C.

126. **Roberts, J. W.** 1969. Termination factor for RNA synthesis. Nature **224**:1168-1174.

127. **Roberts, J. W.** 1993. RNA and protein elements of E. coli and lambda transcription antitermination complexes. Cell **72**:653-655.

128. **Roeder, R. G.** 1991. The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. Trends Biochem.Sci. **16**:402-408.

129. **Rohrmann, G., L. Yuen, and B. Moss**. 1986. Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. Cell **46**:1029-1035.

130. **Rosales, R., N. Harris, B. Y. Ahn, and B. Moss**. 1994. Purification and identification of a vaccinia virus-encoded intermediate stage promoter-specific transcription factor that has homology to eukaryotic transcription factor SII (TFIIS)

and an additional role as a viral RNA polymerase subunit. J.Biol.Chem. **269**:14260-14267.

131. **Rosales, R., G. Sutter, and B. Moss**. 1994. A cellular factor is required for transcription of vaccinia viral intermediate-stage genes. Proc.Natl.Acad.Sci.U.S.A. **91**:3794-3798.

132. **Sanz, P. and B. Moss**. 1998. A new vaccinia virus intermediate transcription factor. J.Virol. **72**:6880-6883.

133. **Sanz, P. and B. Moss**. 1999. Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulates the intermediate stage of viral gene expression. Proc.Natl.Acad.Sci.U.S.A **96**:2692-2697.

134. **Sawadogo, M. and R. G. Roeder**. 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative in vitro assay. Proc.Natl.Acad.Sci.U.S.A **82**:4394-4398.

135. **Sawadogo, M. and A. Sentenac**. 1990. RNA polymerase B (II) and general transcription factors. Annu.Rev.Biochem. **59**:711-754.

136. **Schnapp, G., B. R. Graveley, and I. Grummt**. 1996. TFIIS binds to mouse RNA polymerase I and stimulates transcript elongation and hydrolytic cleavage of nascent rRNA. Mol.Gen.Genet. **252**:412-419.

137. **Schnierle, B. S., P. D. Gershon, and B. Moss**. 1992. Cap-specific mRNA (nucleoside-O2'-)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein. Proc.Natl.Acad.Sci.U.S.A. **89**:2897-2901.

138. **Schwer, B., P. Visca, J. C. Vos, and H. G. Stunnenberg**. 1987. Discontinuous transcription or RNA processing of vaccinia virus late messengers results in a 5' poly(A) leader. Cell **50**:163-169.

139. **Severinov, K.** 2000. RNA polymerase structure-function: insights into points of transcriptional regulation. Curr.Opin.Microbiol. **3**:118-125.

140. **Shi, X., M. Chang, A. J. Wolf, C. H. Chang, A. A. Frazer-Abel, P. A. Wade, Z. F. Burton, and J. A. Jaehning**. 1997. Cdc73p and Paf1p are found in a novel RNA polymerase II-containing complex distinct from the Srbp-containing holoenzyme. Mol.Cell Biol. **17**:1160-1169.

141. **Shilatifard, A.** 1998. Identification and purification of the Holo-ELL complex. Evidence for the presence of ELL-associated proteins that suppress the transcriptional inhibitory activity of ELL. J.Biol.Chem. **273**:11212-11217.

142. **Shilatifard, A., D. Haque, R. C. Conaway, and J. W. Conaway**. 1997. Structure and function of RNA polymerase II elongation factor ELL. Identification of two overlapping ELL functional domains that govern its interaction with polymerase and the ternary elongation complex. J.Biol.Chem. **272**:22355-22363.

143. **Shilatifard, A., W. S. Lane, K. W. Jackson, R. C. Conaway, and J. W. Conaway**. 1996. An RNA polymerase II elongation factor encoded by the human ELL gene. Science **271**:1873-1876.

144. **Shuman, S. and B. Moss**. 1989. Bromouridine triphosphate inhibits transcription termination and mRNA release by vaccinia virions. J.Biol.Chem. **264**:21356-21360.

145. **Simpson, D. A. and R. C. Condit**. 1994. The vaccinia virus A18R protein plays a role in viral transcription during both the early and the late phases of infection. J.Virol. **68**:3642-3649.

146. **Simpson, D. A. and R. C. Condit**. 1995. Vaccinia virus gene A18R encodes an essential DNA helicase. J.Virol. **69**:6131-6139.

147. **Struhl, K.** 1999. Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. Cell **98**:1-4.

148. **Studier, F. W. and B. A. Moffatt**. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J.Mol.Biol. **189**:113-130.

149. **Surratt, C. K., S. C. Milan, and M. J. Chamberlin**. 1991. Spontaneous cleavage of RNA in ternary complexes of Escherichia coli RNA polymerase and its significance for the mechanism of transcription. Proc.Natl.Acad.Sci.U.S.A **88**:7983-7987.

150. **Svejstrup, J. Q., P. Vichi, and J. M. Egly**. 1996. The multiple roles of transcription/repair factor TFIIH. Trends Biochem.Sci. **21**:346-350.

151. **Takagi, Y., R. C. Conaway, and J. W. Conaway**. 1996. Characterization of elongin C functional domains required for interaction with elongin B and activation of elongin A. J.Biol.Chem. **271**:25562-25568.

152. **Tan, S., R. C. Conaway, and J. W. Conaway**. 1995. Dissection of transcription factor TFIIF functional domains required for initiation and elongation. Proc.Natl.Acad.Sci.U.S.A **92**:6042-6046.

153. **Tjian, R. and T. Maniatis**. 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77**:5-8.

154. **Uptain, S. M., C. M. Kane, and M. J. Chamberlin**. 1997. Basic mechanisms of transcript elongation and its regulation. Annu.Rev.Biochem. **66**:117-172.

155. **Wada, T., T. Takagi, Y. Yamaguchi, D. Watanabe, and H. Handa**. 1998. Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II-dependent transcription in vitro. EMBO J. **17**:7395-7403.

156. **Wang, D. and R. Landick**. 1997. Nuclease cleavage of the upstream half of the nontemplate strand DNA in an Escherichia coli transcription elongation complex causes upstream translocation and transcriptional arrest. J.Biol.Chem. **272**:5989-5994.

157. **Woychik, N. A. and R. A. Young**. 1994. Exploring RNA Polymerase II Structure and Function, p. 227-242. *In* R. C. Conaway and J. W. Conaway (ed.), Transcription: Mechanisms and Regulation. Raven Press, New York.

158. **Wright, C. F., A. E. Hubbs, S. K. Gunasinghe, and B. W. Oswald**. 1998. A vaccinia virus late transcription factor copurifies with a factor that binds to a viral late promoter and is complemented by extracts from uninfected HeLa cells. J.Virol. **72**:1446-1451.

159. **Wright, C. F., J. G. Keck, M. M. Tsai, and B. Moss**. 1991. A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. J.Virol. **65**:3715-3720.

160. **Wright, C. F. and B. Moss**. 1989. Identification of factors specific for transcription of the late class of vaccinia virus genes. J.Virol. **63**:4224-4233.

161. **Xiang, Y., D. R. Latner, E. G. Niles, R. C. Condit.** 2000. Transcription Elongation Activity of the Vaccinia Virus J3 Protein in Vivo Is Independent of Poly(A) Polymerase Stimulation. Virology. **269**:356-369.

162. **Xiang, Y., D. A. Simpson, J. Spiegel, A. Zhou, R. H. Silverman, and R. C. Condit**. 1998. The vaccinia virus A18R DNA helicase is a postreplicative negative transcription elongation factor. J.Virol. **72**:7012-7023.

163. **Xie, Z. and D. Price**. 1997. Drosophila factor 2, an RNA polymerase II transcript release factor, has DNA-dependent ATPase activity. J.Biol.Chem. **272**:31902-31907.

164. **Xie, Z. and D. H. Price**. 1996. Purification of an RNA polymerase II transcript release factor from Drosophila. J.Biol.Chem. **271**:11043-11046.

165. **Yamaguchi, Y., T. Takagi, T. Wada, K. Yano, A. Furuya, S. Sugimoto, J. Hasegawa, and H. Handa**. 1999. NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. Cell **97**:41-51.

166. **Yan, Q., R. J. Moreland, C. J. Weliky, and R. C. Conaway**. 1999. Dual roles for transcription factor IIF in promoter escape by RNA polymerase II. J.Biol.Chem. **274**:35668-35675.

167. **Yarnell, W. S. and J. W. Roberts**. 1992. The phage lambda gene Q transcription antiterminator binds DNA in the late gene promoter as it modifies RNA polymerase. Cell **69**:1181-1189.

168. **Zhang, G., E. A. Campbell, L. Minakhin, C. Richter, K. Severinov, and S. A. Darst**. 1999. Crystal structure of Thermus aquaticus core RNA polymerase at 3.3 A resolution. Cell **98**:811-824.

169. **Zhang, Y., B. Y. Ahn, and B. Moss**. 1994. Targeting of a multicomponent transcription apparatus into assembling vaccinia virus particles requires RAP94, an RNA polymerase-associated protein. J.Virol. **68**:1360-1370.

170. **Zhang, Y., J. G. Keck, and B. Moss**. 1992. Transcription of viral late genes is dependent on expression of the viral intermediate gene G8R in cells infected with an inducible conditional-lethal mutant vaccinia virus. J.Virol. **66**:6470-6479.

BIOGRAPHICAL SKETCH

Cari Lynn Aspacher was born on August 12, 1972, in Toledo, Ohio. She spent the first six years of her life in Ohio before moving to Lake Placid, Florida. After only a couple of years, she moved again, to Winter Haven, Florida, where she grew up and continued what would be a long academic career. She enrolled in undergraduate studies at Stetson University in DeLand, Florida in the fall of 1990. She was awarded a Bachelor of Science degree in biology in the spring of 1994. She then moved back to Winter Haven, Florida to contemplate her future and spent a good portion of the year substitute teaching at both the elementary and high school levels. She spent three of those months on a long-term assignment teaching fifth grade. She began her graduate studies in the Department of Molecular Genetics and Microbiology in the fall of 1995. She joined the lab of Richard Condit in the summer of 1996. In October of 1999 she married Daniel Lackner, a fellow graduate student in the Department of Molecular Genetics. In December of 2000 she receives a Ph.D. in biomedical science.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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